THE UNIVERSITY *of* EDINBURGH

Mediators and modulators of immunity to helminths

Kara Jayne Filbey

A thesis submitted for the degree of Doctor of Philosophy

University of Edinburgh

2013

Declaration

I declare that this thesis has been composed by myself, describes my own work and that the work has not been submitted towards any other degree.

The experiments in this thesis on antibody responses to HES (chapter 1) were carried out in close collaboration with Dr James Hewitson, and as such, have been published with joint first authorship (see publication in Appendix).

Selected figures of experiments carried out by other researchers have been used where appropriate, and have been attributed to these authors in the text.

Kara Filbey

August 2013

Acknowledgements

To try and describe these past few years - well, here have been ups and downs, great days and very, very bad days, but there has also been drinking, eating, dancing, conferencing, weddings, babies, scavenger hunts, camping, hiking, whisky tasting, zombies, much more drinking and many other fun times which will keep me smiling and missing you guys for years to come! I thought I knew what I was letting myself in for 4 years ago, but what a journey this ridiculous experience called a PhD has been!

I can't imagine having been able to get through this without the advice and guidance of Rick. You have been such an excellent supervisor, giving encouragement when I've doubted myself and always keeping a straight face when I made ridiculous comments and suggestions (which I think I must have done a lot of the time)! You've allowed me to develop the project in my own way and progress independently, which I'm sure will help me in the future. You also let me to go to some very cool places to conferences! Thank you so much.

James, what to say? You are the most infuriating and exasperating person I've ever worked with! But you have taught me so much, and had unlimited patience with me, and my stupid questions. For this I will be eternally grateful, and you'll be hearing from me for advice and staining panels for the foreseeable future! I hope you find the next exciting stage in your life wonderful, fulfilling and happy.

Right at the start of my time in the Maizels lab, John, Henry and Yvonne taught me invaluable skills in mashing, counting, scraping and infecting things, skills that will always be useful I'm sure, and stood me in good stead for many successful experiments! Thank you also to all the people since then, who have taught me so many things, and been such great people to work with. Special mentions go to Katie and Henry for always being so encouraging and knowledgeable, to Yvonne for being an all round fabulous person, helping with all the thankless tasks and dealing with Nippo for me, to Elaine for taking over the HES and antibody mantle so expertly, to Tara who taught me how to do IHC and let me use a raft of reagents, to Martin Waterfall for his ridiculous FACS knowledge and help, and to Charles, Danielle and especially Esther for

being such able and interested students – it was a pleasure to help teach you about how cool worms are! Thank you also to all the other past and present members of Team Maizels, who have made the lab a wonderful place in which to work, and to all the other people in Ashworth who have imparted wisdom, reagents and chat. Thank you so much to Katie and Lidia who have been wonderful and excellent friends throughout. Also to Katie, and Marieke and Judi, who during my Honours project inspired me to embark on this crazy career-path.

Thank you also to everyone at UCB, especially Tim, Helen, Michael and Karine, who let me loose in their scary industrial labs, gave me lots of advice on the thesis and have been so encouraging and supportive. I have always felt welcome whenever I've come to visit you in sunny Slough! Also, Adam in York for doing all the mass spec, and being patient with me after many emailed queries, and the sectioning people at Little France who expertly mount and slice guts just the way I like them!

Lab life was made much nicer by the eternally sunny faces of Lucy (who I must also thank for amazing cake) and Lisa - you will never get me to appreciate 'young peoples' music, lower-class crisps or other beige food, but you are excellent and I miss singing at the top of my voice and dancing around the lab with you. Lauren and Janice – you have both been the best of friends, without whom my happiness levels, gin intake and general wellbeing would have been 100 times lower, especially in this final year. I miss you coming to the office for hugs L, and will be lost without our 'putting the world to rights' extended coffee breaks J.

Much love to my eternally baffled but always supportive family, and finally to my best friend and partner-in-crime, Frank, who I absolutely, without a doubt, could not have even contemplated doing this without. You have kept me sane, fed, smiling and safe in the knowledge you will always be there to support and help me when times are tough. I can't wait to begin a new adventure with you soon.

This work is dedicated to Douglas and Edith Vaughan, who were always such supportive and loving grandparents.

Thesis abstract

Parasitic helminths infect millions of people and animals worldwide. A key feature of their lifecycle is the longevity of survival within a single host, which is often attributed to the ability of the parasite to divert or modulate the immune response against it. The excretory-secretory (ES) products released by helminths are of interest as the mediators of such immunomodulation. *Heligmosomoides polygyrus* is an excellent model of gastrointestinal (GI) helminth infection in rodents, and has been used here to investigate several aspects of the immune response, and the manipulation of these, in mice.

Firstly, the roles of B cells and antibodies in infection with *H. polygyrus* and towards the adult ES (HES) were investigated. Using several B cell-deficient mouse strains, a minimal effect on immunity to primary infection with H. polygyrus was demonstrated. However, primary infection serum binds to a select set of highly immunodominant components of the complex protein mixture of HES, which were identified as venom allergen-like proteins (VALs).

Utilising four strains of mice that vary in their resistance phenotype to *H. polygyrus*, several aspects of immunity towards the worm were investigated. Increased levels of markers of alternatively activated macrophages, which are a key component of the granulomatous inflammatory response around invading *H. polygyrus* larvae, were found in the most resistant strains, SJL and BALB/c. Depletion of macrophages, by administration of clodronate, severely disrupted the granuloma and parasite clearance. Numbers of innate lymphoid cells and the subsequent Th2 response, specificity range and titre of antibody, and activation of regulatory T cells all correlate with a resistant phenotype.

A deficiency in the cytokine macrophage migration inhibitory factor (MIF) renders a resistant BALB/c mouse completely susceptible to infections with *H. polygyrus*, and *Nippostronygylus brasiliensis*, an acute model of GI helminth infection. This is accompanied by a failure to induce both ILCs and an early myeloid-derived cell population upon infection. The influx of alternatively activated macrophages around

larvae in the mucosa of the small intestine is delayed in $MIF^{-/-}$ mice, although all immunological parameters are comparable to wild-type by day 14 post-infection. The susceptible phenotype of $MIF^{-/-}$ mice can be replicated using a chemical inhibitor of MIF in BALB/c mice.

Finally, the previously documented transforming growth factor-β (TGF-β) activity of HES was dissected out further using two methods of fractionation. Distinct fractions with TGF-β activity were subjected to mass spectrometry to identify protein components that could be potential candidates for this activity.

Lay summary

Parasitic worms, such as hookworms and tapeworms, infect millions of people and animals worldwide. They can survive for many years in a single host without killing it, although some species do cause severe symptoms including tissue damage and anaemia. The ability of worms to survive for such a long time has been attributed to their ability to divert the host's immune response away from them, using various mechanisms including secreting molecules that change the way the body responds to the infection.

People respond to worm infections in different ways. Some are resistant to infection and some can have severe symptoms, depending on their genetics and the way their immune systems cope with parasites. We have used several different types of mice, with different genetics, to investigate the immune responses that are needed to mount a successful immune response against parasitic worms. These include the antibodies and cells that the immune system is comprised of. Knowing which genes and immune responses are involved in resistance to parasites could lead to better vaccines and drugs for humans and livestock.

We have also looked at the molecules that are secreted by worms, and have found several candidates that could potentially change the immune response so as to allow the worm to live for longer without being killed by the immune system. Making drugs that eliminate these molecules could be a way to cure parasitic diseases in humans.

Abbreviations

1° / 2° - primary / secondary

4-IPP - 4-iodo-6-phenylpyrimidine

AAMΦ – alternatively activated macrophage

AID – activation induced deaminase

APC - antigen presenting cell

Arg1 – arginase 1

ASP – ancylostoma-secreted protein

BAL(F) – bronchoalvelolar lavage (fluid)

BCR – B cell receptor

Breg – regulatory B cell

CAMΦ – classically activated macrophage

CCL – chemokine (CC motif) ligand

Chi3L3 – chitinase-3-like-3 (or Ym1)

CPI – cysteine protease inhibitor

DC – dendritic cells

DMSO – dimethyl sulphoxide

DTR – diphtheria toxin receptor

EAE – experimental autoimmune encephalitis

ELISA – enzyme-linked immunosorbent assay

ES - excretory-secretory products

EST – expressed sequence tag

FACS – fluorescence assisted cell sorting

FCS – foetal calf serum

FIZZ1 – found in inflammatory zone 1

GI – gastrointestinal

GM-CSF – granulocyte-macrophage colony-stimulating factor

HES – *H. polygyrus* ES

HEX (or AWH) – *H. polygyrus* adult worm extract/homogenate

HRP – horseradish peroxidase

i.n. - intra-nasal

i.p. – intra-peritoneal

ICOS – inducible T cell costimulator

IEC – intestinal epithelial cell

IEL – intra-epithelial lymphocyte

IFN – interferon

Ig - immunoglobulin

IL – interleukin

ILC – innate lymphoid cell

KLH – keyhole limpet hemocyanin

LC-MS/MS – liquid chromatography with tandem mass spectrometry

LN – lymph node

LPS – lipopolysaccharide

mAb – monoclonal antibody

MBP – major basic protein

MCP – mast cell protease

MDSC – myeloid-derived suppressor cell

MHC – major histocompatibility complex

MIF – macrophage migration inhibitory factor

MLN – mesenteric lymph node

MUC – mucin

MW – molecular weight

NeMΦ – nematode-elicited macrophage

NES – *N. brasiliensis* ES

NO / NOS – nitric oxide / nitric oxide synthase

NSN – novel secreted protein without signal peptide

NSP – novel secreted protein

OVA – ovalbumin

PCR – polymerase chain reaction

PL – peritoneal lavage

PP – Peyer's patches

PRR – pattern recognition receptor

RACE – rapid amplification of cDNA ends

RBC- red blood cell

RELM – resistin-like molecule

ROS – reactive oxygen species

rpm – revolutions per minute

SCID – severe combined immune-deficient

SCP – sperm coating protein

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEA – *Schistosoma* soluble egg antigen

SEAP – secreted alkaline phosphatase

STAT – signal transducers and activators of transcription proteins

TCR – T cell receptor

TFF2 – trefoil factor 2

T_{FH} – T follicular helper cells

TGF – transforming growth factor

Th – T helper cell

TLR – Toll like receptor

TNF – tumour necrosis factor

Treg – regulatory T cell

TSLP – thymic stromal lymphopoietin

VAL – venom allergen–like protein

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Introduction

1. HELMINTH INFECTIONS

Parasitic worm (helminth) infections in humans are still widespread in the developing world, and are the cause of high morbidity, mortality, and developmental and cognitive defects (Crompton 1999; Lustigman *et al* 2012). Highest prevalence is seen in children and the cause of growth stunting, impaired memory and learning skills, and in pregnant women, low birth weight of the child and higher risk of maternal morbidity (Hotez *et al* 2008). As well as their direct effects, helminths can have a detrimental impact on coinfection outcomes and on the treatment of these diseases (Urban *et al* 2007; Ezenwa and Jolles 2011). There are still many questions regarding effectiveness and sustainability of anti-helminth treatments, and as yet no effective vaccine, although some research initiatives have recently been set up (Hotez *et al* 2008). However, the mechanisms involved in the initiation and implementation of host immunity to helminths must be more fully understood before successful vaccinations can be developed (Harris 2011).

Improved hygiene, less crowded living conditions, smaller household size, and improved healthcare provision since the 1950s in economically developed countries has seen a near elimination of endemic worm infection in the general population. Conversely, a high incidence of allergic and autoimmune disease is now found in these countries, which are much less prevalent in the developing world (Bach 2002). Increased patient numbers with asthma, atopic dermatitis, multiple sclerosis, Crohn's disease, type 1 diabetes and other inflammatory conditions have been widely documented in Europe and North America (Elliott and Weinstock 2012). The inverse correlation of helminth infections with atopic and autoimmune disease development is further supported by studies including those that found higher incidence of Crohn's disease (Weinstock *et al* 2002) and allergic skin test reactivity (Lynch *et al* 1993) after deworming regimes.

These epidemiological trends, and the high prevalence and longevity of parasitic diseases in otherwise immunocompetent hosts has led to the in-depth examination of possible mechanisms involved in this phenomenon (Maizels *et al* 1993; Maizels and Yazdanbakhsh 2003; Hoerauf *et al* 2005). It is now clear that some atopic and autoimmune disease are driven by particular arms of the immune system, and the skewing or regulation of these mechanisms by the presence of a helminth parasite – either by direct or indirect means – is the focus of intense research and development of potential therapeutics for the aforementioned debilitating conditions (Harnett and Harnett 2010; Osada and Kanazawa 2010; Khan and Fallon 2013).

1.1 Models of helminth infection

Helminths are classified in two distinct phyla – Nematoda (roundworms) and Platyhelminthes (flatworms). The Platyhelminthes are further split into trematodes (which include the flukes such as *Schistosoma* and *Fasciola* species) and cestodes (tapeworms such as *Taenia* and *Echinococcus* species)(Brindley *et al* 2009). The nematodes include a variety of roundworms including filarial species such as *Brugia* and *Onchocerca*, hookworms such as *Nippostrongylus, Necator* and *Ancylostoma,* and others such as *Trichinella* and the whipworm *Trichuris* (Brindley *et al* 2009). The suborder Trichostrongylina contains several important helminths important in human health and studied in murine models, such as *Haemonchus contortus*, *Teladorsagia circumcincta* and *Heligmosomoides polygyrus* (Gouÿ de Bellocq *et al* 2001). Some of these helminth infections are associated with well-known major human pathologies and disabilities, such as elephantiasis caused by lymphatic filariasis, blindness caused by filarial worms that migrate through the eye, severe anaemia caused by hookworms and liver, urinary tract and intestinal pathology and fibrosis caused by schistosomiasis (Brindley *et al* 2009; Periago and Bethony 2012; Toledo *et al* 2012).

H. polygyrus naturally infects wild mice, and is widely used as a model for chronic gastrointestinal nematode infection in laboratory mice. Previously named *Nematospiroides dubius* (Behnke *et al* 1991), it has since been suggested that it be

renamed again to *H. bakeri* to distinguish the laboratory strain from that found in wild populations (Behnke and Harris 2009; Behnke and Harris 2010), although this has not met with universal support from other researchers and so it is still commonly referred to as *H. polygyrus bakeri,* or *H. polygyrus* (Maizels *et al* 2010). This worm presents an excellent and easily maintained model for researchers and has been widely published on for several decades (Behnke *et al* 2009; Maizels *et al* 2011; Reynolds *et al* 2012).

Infective *H. polygyrus* L3 larvae enter the mouse orally (by oral gavage in laboratory models), and invade the duodenal mucosa within 24 hours of ingestion (see Fig 1). Here they moult through two larval stages and after around 8 days emerge as adults into the intestinal lumen, where they wrap tightly around villi to keep in place (Telford *et al* 1998). Fluorescent staining of different compartments of the body revealed that the epithelial cell layer of the intestine is the most likely food source of adult *H. polygyrus* (Bansemir and Sukhdeo 1994), which may correlate with areas of the small intestine with longer villi, which would provide a richer food source (Bansemir and Sukhdeo 1996). The lumen is also the site for mating, which results in egg production into the faeces of the mouse. Hence, both egg counts from faecal pellets, and enumeration of adult worms in the lumen of the gut, are the main ways of measuring parasite persistence and fitness (Reynolds *et al* 2012).

In order to collect ES from adult *H. polygyrus,* parasites are removed from the small intestine of mice infected with 400-500 L3 larvae 14 days previously. Worms are separated from mucus and intestinal contents over a Baermann apparatus consisting of a glass funnel attached to a glass collection vial with plastic tubing. The apparatus is filled with warmed HBSS and gut contents placed in muslin bags which are lowered into the media, and left at 37°C for 2 hours to allow worms to settle in the collection vial. After several washes they are placed in flasks in serum-free RPMI, and left at 37°C for 21 days, media being collected, and fresh media added, every 2-3 days throughout. HES is pooled and run over a 3000 MW cut-off membrane for concentration under pressure, in an Amicon unit (see Fig 2). Once sterile-filtered, HES is frozen at -80°C until used.

Nippostrongylus brasiliensis is a rodent model of acute helminth infection, as the majority of immunocompetent mouse strains can clear this parasite within 9-14 days (Camberis *et al* 2003). Infective L3-stage larvae enter the host via the skin (or subcutaneous injection in experimental models) and migrate to the lung, via the microvasculature, within 15 hours (Ogilvie and Jones 1971)(see Fig 3). Maturation occurs in the lung, and fourth-stage larvae are transported by ciliary action and mucus up the trachea, and into the oesophagus (Harvie *et al* 2013), where they are ingested and transported to the small intestine, for the most part by day 3 post-infection (Ogilvie and Jones 1971). Maturation to adult worms occurs in the duodenum, where mating and egg production, from day 6 post-infection, proceed until worms are expelled. As with *H. polygyrus*, luminal worm and faecal egg counts are used to assess the effectiveness of immunity towards the parasite. Through fluorescent staining of host ingesta, blood and tissue, the food source of adult worms is thought to be host intestinal tissue (Bansemir and Sukhdeo 2001).

H. polygyrus and *N. brasiliensis* are 2 very different models of gastrointestinal (GI) helminth infection, with *H. polygyrus* remaining in the GI tract throughout its lifecycle, and inducing minimal pathology, compared to the extensively migratory nature of *N. brasiliensis* resulting in long-term architectural and immunological disruption in the lung tissue (Marsland *et al* 2008; Reece *et al* 2008). Although the immune responses to these parasites have much in common, the nature of local immune mechanisms, and those required for clearance of adult worms, are quite different. Both parasites also induce extremely effective memory responses to reinfection (Wahid and Behnke 1992; Gause *et al* 2003; Harvie *et al* 2013) and as such, they are excellent tools in the study of innate and adaptive immune responses and mechanisms involved in clearance of parasitic helminths.

Figure 1. Lifecycle *of H. polygyrus*

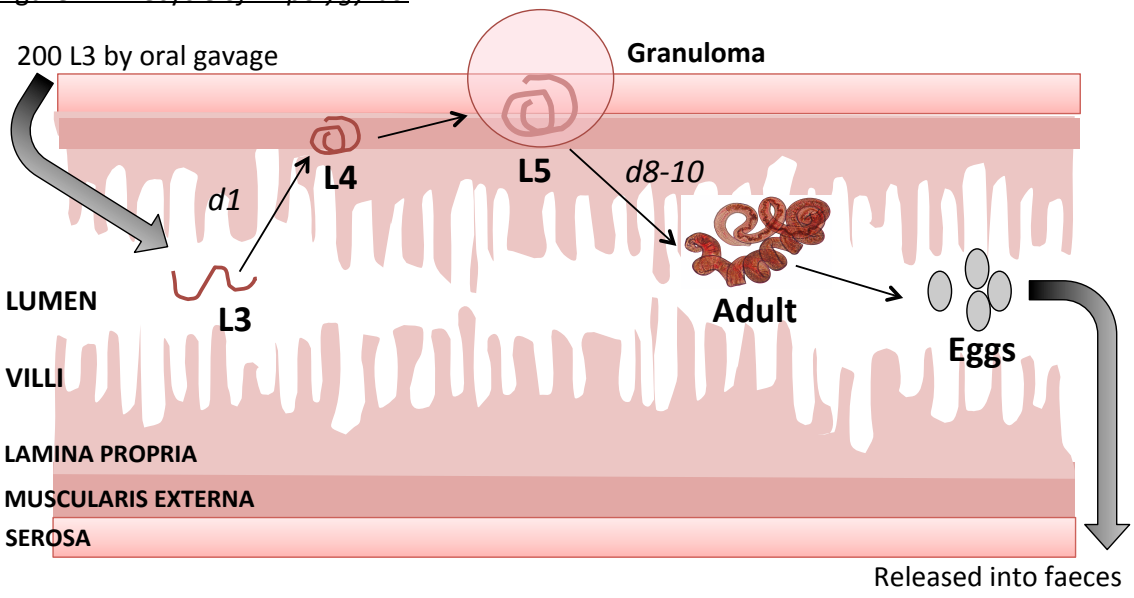


Figure 2. Preparation of HES and *H. polygyrus* larvae for infections

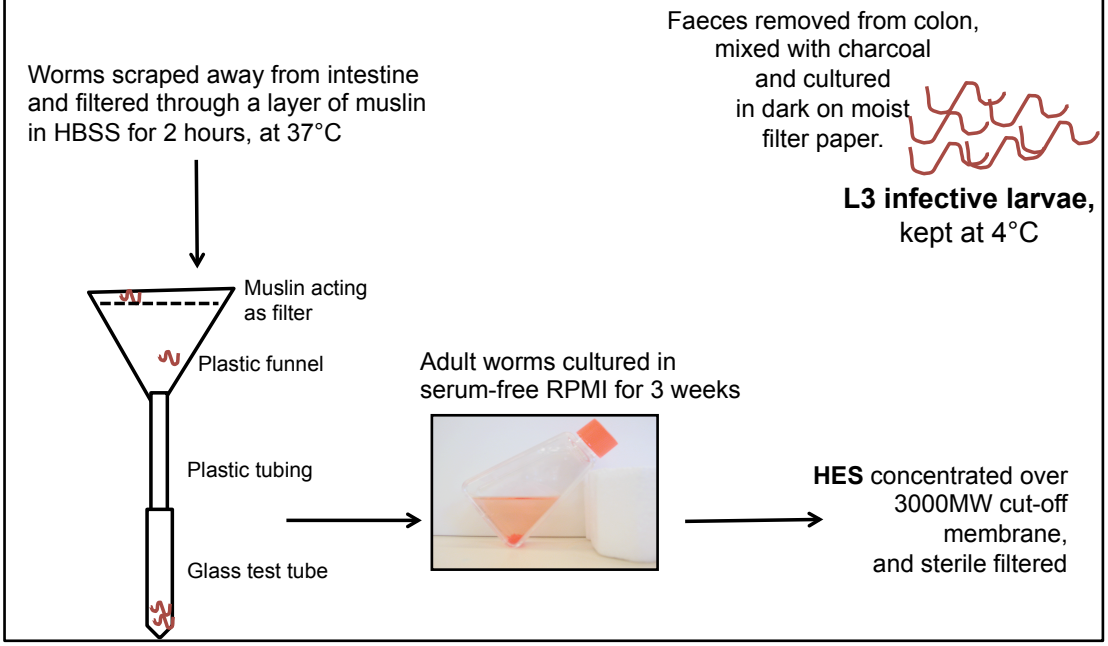
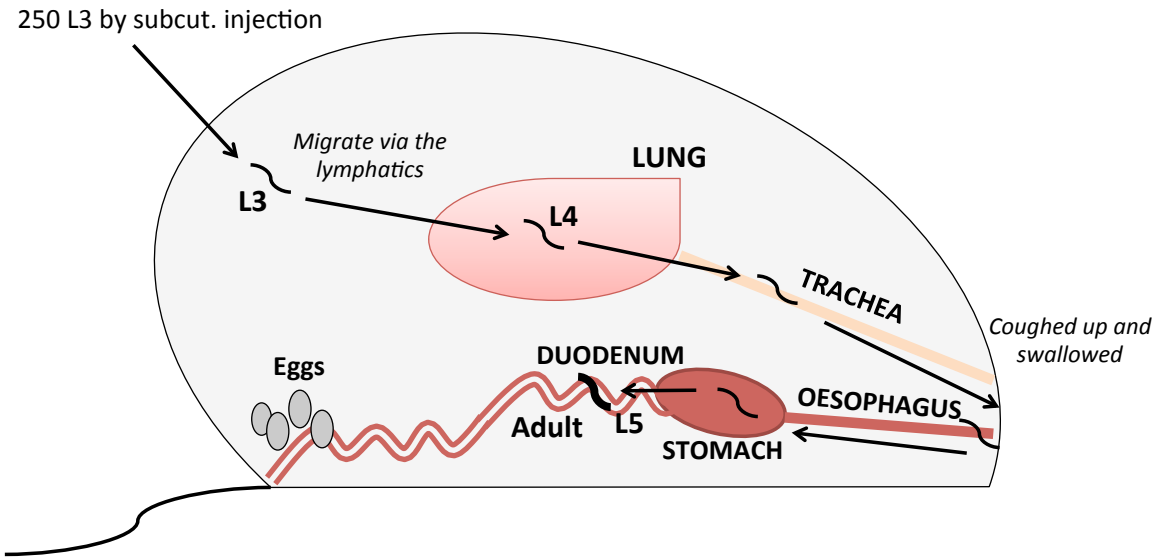


Figure 3. Lifecycle of *N. brasiliensis*



1.2. Immunomodulation and Coinfection

It is now widely accepted that certain parasites can modulate the host's immune response to prolong their own survival, thus increasing their chances of reproducing and continuing the lifecycle forward (Maizels *et al* 1993; Maizels and Yazdanbakhsh 2003; McSorley *et al* 2013). This down-regulation of the immune response against parasites is often also manifested in a reduced response to 'bystander antigens' (Greene *et al* 1983; Terrazas *et al* 2010a), vaccines (Sabin *et al* 1996; Cooper *et al* 1998), self-antigens in autoimmune diseases (Elliott and Weinstock 2012), transplants (Ledingham *et al* 1996; Liwski *et al* 2000; Li *et al* 2011a) and allergens (Wilson and Maizels 2004; Danilowicz-Luebert *et al* 2011; Feary *et al* 2011). Probably most relevant, in endemic areas, is the effect of helminths on the outcome of co-infections.

Malaria is frequently co-endemic with human helminthiases. Studies in Africa and Asia report varied results, with some showing helminth infection correlating with higher incidence of malaria (Nacher *et al* 2002; Degarege *et al* 2012), and others a protective effect of helminths with regard to malaria outcome (Murray *et al* 1978; Nacher *et al* 2000). Evidence that helminth infection could negatively alter antibody responses to *Plasmodium* candidate vaccine antigens is also a concern for malaria control programmes (Esen *et al* 2012). Mouse models illustrate the variability of responses based on *Plasmodium* strain and the type of malarial disease caused (Knowles 2011) – helminth co-infection exacerbates disease parameters and parasite transmission in ordinary resolving *Plasmodium* strains such as *P. chabaudi* and *P. yoelii* (Noland *et al* 2007), but death is significantly delayed in mice with the cerebral form, *P. berghei* ANKA (Knowles 2011).

There is a general consensus that helminth infections negatively impact on the human immune response against *Mycobacterium tuberculosis* (Elias *et al* 2007; Resende Co *et al* 2007; Abate *et al* 2012), possibly through reduced T cell responsiveness and lower Th1 cytokine responses (Resende Co *et al* 2007). Due to the high number of complicating co-infections in HIV infected people, it is hard to determine the specific impact of helminths on them, but several reviews of the literature suggest that deworming maybe of benefit in the reduction of viral load

(Borkow and Bentwich 2006; Walson and John-Stewart 2007). Co-infection with several helminths at once is common in endemic areas (Hotez *et al* 2008) and outcomes are variable in human studies. Some suggest that antibodies to one helminth can be cross-reactive with another, and therefore be protective against both worms (Corrêa-Oliveira *et al* 1988). Others, that the response to hookworms can be down-modulated by the presence of *Schistosoma mansoni* (Corrêa-Oliveira *et al* 2002).

There are a number of studies involving co-infection in mouse models, particularly with *H. polygyrus,* which shed some light on the immune responses it engenders. In terms of other helminth infections, the usually short-lived infection of *N. brasiliensis* is prolonged with concurrent *H. polygyrus* (Colwell and Wescott 1973); conversely, pre-infection with *H. polygyrus* reduces liver pathology associated with *S. mansoni* infection, and correlates with reduced inflammatory cytokine levels and an increase in *Chi3l3* (Ym1) and *TGFB1* (TGF-β) transcripts (Bazzone *et al* 2008). Infection with protozoan parasites (*Trypanosoma congolense* and *Eimeria falciformis*) in the first week of *H. polygyrus* infection leads to a less effective anti-protozoal response, increased replication of parasites and increased morbidity (Fakae *et al* 1994; Rausch *et al* 2010). In *Eimeria* infection this corresponds with reduced anti-protozoal Th1 responses, and a higher Treg activation when early stage *H. polygyrus* is present, but not at a later stage in the lifecycle (Rausch *et al* 2010). When *Toxoplasma gondii* is administered at the same time as *H. polygyrus* there is increased survival of the mice and reduced intestinal pathology compared to infections with *T. gondii* alone (Khan *et al* 2008). *H. polygyrus* exacerbates infection with *Cryptosporidium parvum* with inhibition of the necessary Th1 response (Bednarska *et al* 2008).

The picture is clearer in malaria and bacterial infections where a protective response has been shown to be highly Th1 orientated, and a skew towards Th2 responses by *H. polygyrus* severely compromises protection. With various strains of *Plasmodium*, *H. polygyrus* consistently causes higher morbidity and parasitaemia, correlated with down-regulated IFN-γ responses and high IL-10 and TGF-β levels (Su *et al* 2005; Noland *et al* 2008; Tetsutani *et al* 2009). The outcome of *Citrobacter rodentium* bacterial infection is compromised with concurrent *H. polygyrus* infection, with

higher bacterial loads and translocation across the intestinal wall leading to severe colitis (Weng *et al* 2007). Several studies have found Th2-mediated alternative activation of macrophages in this setting to contribute to less effective bacterial killing and clearance (Weng *et al* 2007; Su *et al* 2012).

The studies outlined above point to roles for the skewing of the immune response to a Th2 type response and the induction of regulatory cells and cytokines as the major factors involved in immunomodulation by *H. polygyrus*. Regulation by helminths of the immune responses against them, and the limiting of immunopathology that might otherwise compromise the fitness of their hosts, is a cornerstone of the success of these parasites (Maizels *et al* 1993; Hoerauf *et al* 2005). Human filarial infections gave evidence of helminth induced regulatory mechanisms, firstly by observations of high levels of the regulatory cytokines IL-10 and TGF-β in these patients (King *et al* 1993; Mahanty *et al* 1996; Doetze *et al* 2000). Furthermore, culture of their cells *in vitro* whilst neutralising these cytokines re-established T cell proliferation (King *et al* 1993).

2. ANTI-HELMINTH IMMUNITY

A key feature of helminth infections in general is the remarkable ability for longevity in a single host, which is attributed to their ability to evade and modulate the immune system (Maizels and Yazdanbakhsh 2003; Maizels *et al* 2004; Hoerauf *et al* 2005; Hewitson *et al* 2009; Ludwig-Portugall and Layland 2012; McSorley and Maizels 2012; McSorley *et al* 2013). As multicellular, often macroscopic, pathogens, the immune responses to helminths are complex and multifaceted (Gause *et al* 2003; Anthony *et al* 2007; Allen and Maizels 2011; Maizels *et al* 2012; Taylor *et al* 2012), and still have many aspects that are not fully understood. However, the central feature of infections with all helminth parasites is the expression of a T-helper 2 (Th2) response, which is characterised by an array of specialised molecules and cells, both innate and adaptive, and the induction of immunoregulatory mechanisms.

2.1 Cytokine responses

In 1986, the formal demonstration that murine $CD4^+$ T cells could be split into subsets according to their cytokine profile led to a new paradigm in immunology, which has since been the basis of the understanding of immune responses and immunopathologies (Mosmann *et al* 1986; Liew 2002). Using antigen-specific T cell clones, cells were catagorised in accordance with their cytokine output upon stimulation and the antibody response they induced in B cells. T helper 1 cells (Th1) produced IL-2, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3, and promoted antibody class-switching to IgG2a, whilst Th2 cells produced IL-3, IL-4 and IL-5, and induced IgE and IgG1 (Mosmann *et al* 1986; Stevens *et al* 1988). The differentiation of human T cell subsets was demonstrated when Th1 cells were induced *in vitro* by stimulation with a purified protein derivative of *M. tuberculosis,* and Th2 by the excretory/secretory products of the helminth *Toxocara canis* (Del Prete *et al* 1991). Since then it has been clearly shown that, in general, type 1 immunity is necessary for protection against bacterial and protozoan parasite infections, whereas type 2 is associated with protection against helminths, and the development of allergic responses (Jankovic *et al* 2001; Liew 2002).

Studies on intracellular parasite *Leishmania major* were instrumental to the discovery of reciprocal regulation of protective immunity through Th1 and Th2 cytokines in mice. Whilst both BALB/c and C57BL/6 mice are susceptible to experimental infection with this parasite, C57BL/6 mice resolve infection and develop long-lasting immunity, whereas BALB/c mice develop non-healing lesions and eventually succumb to infection (Reiner and Locksley 1993). The ability to control infection in C57BL/6 mice was found to be associated with IFN-γ production, and the neutralisation of IL-4 in BALB/c rendered them able to control the infection and survive (Heinzel *et al* 1989). Conversely, it has been demonstrated that mice genetically predisposed to making a Th1 response are more susceptible to a variety of helminths (Wahid *et al* 1994; Behnke *et al* 2003a; Brown *et al* 2003; Dehlawi and Goyal 2003). These findings formed the key conceptual platform from which to

investigate immune parameters associated with resistance to pathogens, and from which to design suitable future experiments.

T cell differentiation occurs in response to several stimuli, including the local cytokine milieu, which is itself directed by innate cells and antigen-presenting cells (APCs) which first encounter the immune stimulus (Kapsenberg 2003). The cytokines produced by Th subsets also act in an autocrine fashion to further amplify those responses and inhibit others (Gajewski and Fitch 1988). The strength of signalling through the TCR, and the antigen dose, have significant effects on the mode of differentiation, as does the co-stimulation provided by APCs (Tao *et al* 1997; O' Garra *et al* 2011). In terms of Th2 differentiation, IL-4 was recognised early on to promote Th2 responses (Le Gros *et al* 1990b; Swain *et al* 1990), and it was subsequently shown to act through the activity of STAT6 (Kaplan *et al* 1996; Takeda *et al* 1996) to promote induction of the transcription factors c-maf and GATA-3 (Ho *et al* 1996; Zheng and Flavell 1997; Murphy and Reiner 2002).

The importance of IL-4, and IL-13, which signal through a common receptor chain (IL-4Rα), in protective immune responses against many helminths, has been clearly demonstrated (Finkelman *et al* 1997; Finkelman *et al* 2004). Although most helminth infections provoke the upregulation of a characteristic set of cytokines and cells, the mechanisms and exact components of the Th2 response needed for the expulsion of adult worms, limiting of egg production, and prevention of damage and pathology, differ for each parasite.

Primary *H. polygyrus* infection induces *Il3, Il4, Il5* and *Il9* gene expression in the mesenteric lymph nodes (MLN) and Peyer's patches (PP) (Svetić *et al* 1993) and elicits the release of high concentrations of IL-4, IL-5, IL-9, IL-10 and IL-13 protein from MLN, spleen and lamina propria cells cultured with parasite antigens (Finney *et al* 2007; Setiawan *et al* 2007; Rausch *et al* 2008). These same cytokines are also produced by MLN cells from *N. brasiliensis*-infected mice, peaking at day 10 post-primary infection (Fallon *et al* 2002). IL-4 is the most important single cytokine for protection against primary and secondary *H. polygyrus* infection, as measured either by expulsion of adult worms or inhibition of their egg production (Urban *et al* 1991b). Treatment of both wild-type BALB/c mice and severe combined immune-

deficient (SCID) mice with IL-4-complex (to prolong the life of the cytokine *in vivo*) abrogates both *H. polygyrus* and *N. brasiliensis* survival, illustrating that IL-4 can mediate protection from helminths, independently of the adaptive immune system (Urban *et al* 1995). However, *Il4*$^{-/-}$ mice can still expel *N. brasiliensis* parasites, indicating that IL-13 is more important than IL-4 in this model (Urban *et al* 1998). Indeed, treatment of mice with an IL-13-antagonist, was more potent than α-IL-4 mAb at protecting against *N. brasiliensis* survival (Urban *et al* 1998). However, in the absence of IL-13, IL-5 and IL-9, IL-4 can alone induce expulsion of *N. brasiliensis* (Fallon *et al* 2002), illustrating the compensatory effect of some cytokines in the absence of others in the set.

IL-4 has potent effects on numerous cell types and processes important in anti-helminth immunity (as described in more detail below) including Th2 cell differentiation, alternative activation of macrophages, intestinal smooth muscle hypercontractility and B cell class switching. IL-4 can even compensate for the absence of cytokines thought to be more important to mastocytosis (IL-9), eosinophilia (IL-5) and goblet cell hyperplasia (IL-9 and IL-13), at least in the setting of *N. brasiliensis* infection (Fallon *et al* 2002). Although IL-13 shares many functions with IL-4, it does have non-redundant roles, demonstrated in the differing responses to *T. muris* in *Il4*$^{-/-}$ and *Il13*$^{-/-}$ mice. Without IL-4, mice could not make a substantial Th2 response, including reduced cytokine and antibody levels compared to wild-type mice, and could not expel the parasite, but *Il13*$^{-/-}$ mice are also susceptible to infection, even in the presence of a strong Th2 response (Bancroft *et al* 1998). This illustrates that IL-4 exerts a more critical effect than IL-13 on Th2 cell development and expansion, while IL-13 is implicated in downstream effects on innate effector mechanisms, such as epithelial cell turnover, involved in expulsion of worms in the *Trichuris* model (Klementowicz *et al* 2012).

IL-5 is also highly upregulated in many helminth infections, but compared to the vital roles demonstrated for IL-4 and IL-13, this cytokine is not as critical to the parasitological outcome in most models. IL-5 has long been known to promote the differentiation, proliferation and chemotaxis of eosinophils (Yamaguchi *et al* 1988). Administration of neutralising antibody to IL-5 (TRFK-5) completely suppresses

parasite-induced blood and tissue eosinophilia during primary infections with *N. brasiliensis* (Coffman *et al* 1989), *H. polygyrus* (Urban *et al* 1991b) and *S. mansoni* (Sher *et al* 1990) but has no detrimental effect on the ability of mice to expel primary infections with these parasites. IL-5 deficiency also has little effect on the outcome of a primary infection with *Toxocara canis* (Takamoto *et al* 1997) or *Trichinella spiralis*, however, expulsion of worms after a secondary *T. spiralis* or *N. brasiliensis* challenge infection is impaired in *Il5*$^{-/-}$ mice, illustrating the differential effects of eosinophils in these settings (Vallance *et al* 2000; Knott *et al* 2007). The same is true for *Litomosoides sigmodontis,* where expulsion of a primary infection is unaffected by IL-5 deficiency, but vaccine-induced immunity is compromised (Le Goff *et al* 2000).

Apart from directly promoting eosinophilia, IL-5 has also been shown to promote neutrophilia indirectly. Administration of α-IL-5 *in vivo* ablated eosinophils and neutrophils, and the formation of inflammatory nodules around *L. sigmodontis* larvae in the thoracic cavity, thus promoting parasite survival (Al-Qaoud *et al* 2000). Neutrophils do not have an IL-5-receptor, but it was found that levels of other cytokines known to promote neutrophilia (IL-8, G-CSF, TNF-α) were strongly reduced in α-IL-5-treated mice. IL-5 is also a B cell growth factor (Sanderson *et al* 1986), particularly for B cells that produce autoantibodies, and induces class switching to IgA (Finkelman *et al* 1990).

Very recently, there has been great interest in discovering the earliest initiators of Th2 responses. Focus has been on innate cells as the local, rapid producers of cytokines and chemokines that shape the subsequent activation status of other cells such as macrophages and DCs, which then in turn help to activate and direct T cell and B cell responses. A cohort of epithelial cell-derived cytokines have been demonstrated to induce type-2 innate lymphoid cells (ILC2s), which produce IL-5 and IL-13 to polarise the response towards Th2, early on in a helminth infection or allergic challenge (Paul 2010; Paul and Zhu 2010; Okoye and Wilson 2011). Other innate cell populations, such as basophils, have also been found to secrete high levels of IL-4 in the tissue, and act as APCs, after sensing of helminth antigens and allergens (Paul and Zhu 2010; Voehringer 2013). These, and other cell types and

mediators involved in responses to helminths, in particular to *H. polygyrus* and *N. brasiliensis*, are described below.

2.2 T cell responses

As described above, the development of $CD4^+$ T cells into Th2 over Th1 is controlled by several different stimuli, and is highly dependent on the signals received from the APC involved (see section 2.3 below). $CD4^+$ cells are crucial to anti-helminth immunity (Gause *et al* 2003; Allen and Maizels 2011). Administration of α-CD4 during primary infection with *H. polygyrus* resulted in increased fecundity of female worms and a significant drop in IgE production, whereas in secondary infection, protective immunity was ablated (Urban *et al* 1991a). Inhibition of worm expulsion was also seen after α-CD4-treatment of mice infected with *T. muris* (Koyama *et al* 1995) and *Angiostrongylus cantonensis* (Aoki *et al* 1998), and blockade of CD4 had a detrimental effect on protective immune responses induced by immunisation with irradiated larvae of *Brugia pahangi* (Bancroft *et al* 1994). However, neither α-CD4 treatment of mice normally resistant to *Brugia malayi*, nor disruption of the gene encoding CD4 in these mice, could render them susceptible, indicating that $CD4^+$ cells are not required for protection against these parasites (Rajan *et al* 1994). In contrast, α-CD8 treatment has little to no effect on the outcome of helminth infection (including *H. polygyrus*) or larval immunisation (Urban *et al* 1991a; Bancroft *et al* 1994; Koyama *et al* 1995; Aoki *et al* 1998). Indeed, IFN-γ-producing $CD8^+$ T cells are associated with susceptibility to and chronicity of *T. muris* infections in mice, although depletion of these cells *in vivo* does not have an effect on worm expulsion, a phenomenon thought to come about by $CD4^+$ T cell compensation (Humphreys *et al* 2004).

Further studies have delineated the co-stimulatory signals required to mount Th2 responses to *H. polygyrus* infection. Blocking signalling through both CD80 (B7-1) and CD86 (B7-2), the ligands on APCs that bind T cell CTLA-4 and CD28 for costimulation, had a broad effect on Th2 responses, with downregulation of primary *H. polygyrus*-induced Th2 cell expansion, IL-4 secretion, eosinophilia, intestinal

mastocytosis and increases in IgG1 and IgE levels (Lu *et al* 1994; Greenwald *et al* 1997). *CD86*$^{-/-}$ mice were not defective in *H. polygyrus*-induced immune responses early in infection (day 6) but by day 14 showed inhibited T cell cytokine secretion and antibody titres (Greenwald *et al* 1999), showing that CD86 is not required for the initiation of an immune response but is needed for the progression and persistence of the T cell response. Although both CD80 and CD86 normally ligate to T cell CD28, *CD28*$^{-/-}$ mice were found to have no impairment in the early CD4^{+}IL-4^{+} response, indicating an alternative mechanism for Th2 co-stimulation in *H. polygyrus* infection (Gause *et al* 1997). In a secondary *H. polygyrus* infection, blocking signalling through CTLA-4 had no effect on protective immunity, suggesting that memory T cells do not need CD28-B7 interactions for their activation to effector cells after a challenge infection (Gause *et al* 1996; Ekkens *et al* 2002).

Mice deficient in another costimulatory molecule, OX40L, showed a specific defect in T cell IL-4 production, and associated IgE production, following *H. polygyrus* infection, which was not accompanied by an effect on Th2 cell expansion, germinal centre formation or IgG1 production (Ekkens *et al* 2003).

Although much work on the specificity of antibodies and B cell receptors (BCRs) in helminth infections has been completed (see section 2.15 below), the specificity of T cell receptors (TCRs), and immunodominant T cell epitopes of helminths are still largely undetermined, other than a requirement for a broad TCR repertoire for *N. brasiliensis* expulsion (Seidl *et al* 2011).

Cells that have γδ TCR chains represent a very different population of T cells to conventional αβ T cells, and most do not possess CD4 or CD8 molecules or recognise antigen through interation with MHC (Kabelitz *et al* 2005). Little research has been done on the importance of γδ TCRs in gastrointestinal helminth infection, even though they have been shown to have a vital role in intestinal epithelial homeostasis and defence, and make up a large proportion of intestinal intraepithelial lymphocytes (IELs) (Komano *et al* 1995; Kabelitz *et al* 2005; Komori *et al* 2006). γδ T cells can generate both Th1- and Th2-like responses, depending on the antigenic stimulus available, and in the peritoneal cavity make IL-4 in response to *N. brasiliensis* infection (Ferrick *et al* 1995). A later study showed that IEL γδ T cells

are a major source of IL-4, IL-5 and IL-13 in *N. brasiliensis* infection (Inagaki-Ohara *et al* 2011). Remarkably, TCR-$\delta^{-/-}$ mice are highly susceptible to this parasite with the majority of mice dying within 3 weeks of infection. Th2 responses were compromised in these mice, with intestinal mastocytosis, Th2 cytokine production from intestinal T cells and goblet cell hyperplasia all reduced and delayed, compared to wild-type mice (Inagaki-Ohara *et al* 2011). However, caution must be taken extrapolating results from mice to humans, as they possess a different repertoire of TCR$\gamma\delta$ genes and are compartmentalised in different areas of the body in these species (Hayday 2000).

It has more recently become clear that there are several other T cell subsets that produce other sets of cytokines and express different transcription factors (Oestreich and Weinmann 2012). Furthermore, it is apparent that T cell subsets may display a degree of plasticity with regards to conversion into a different phenotype and coexpression of markers (Bluestone *et al* 2009; Murphy and Stockinger 2010; Cannons *et al* 2013). One example is the recently described Th9 subset, closely related to Th2 cells in being IL-4-dependent but induced by TGF-β to switch to IL-9 and adopting a Th9 phenotype, distinct from other T cell subsets previously described (Veldhoen *et al* 2008). However, Th9 cells also make IL-10, illustrating the overlap in phenotype between subsets and the difficulty of compartmentalizing this complex area of immunology (Stassen *et al* 2012). The specific role of IL-9, and Th9 cells, in protection against helminths is unresolved. Levels of IL-9 correlate with protection against helminths in mice and humans, along with other Th2 signature cytokines (Else *et al* 1993; Wahid *et al* 1994; Turner *et al* 2003) and IL-9 over-expressing mice are more immune to *H. polygyrus* (Hayes *et al* 2004) and *T. spiralis* (Faulkner *et al* 1997). It has been well characterized in *Trichuris muris* infection, where it has been found to promote mastocytosis, antibody class switching to IgE, eosinophilia, intestinal smooth muscle contractility and resulting worm expulsion (Faulkner *et al* 1998; Richard *et al* 2000; Khan *et al* 2003). In *N. brasiliensis* infection however, IL-9-deficiency does not impair the ability of mice to expel adult worms (Townsend *et al* 2000a), even though it has a key role in the development of mastocytosis in this model (Fallon *et al* 2002).

T-follicular helper cells (T_{FH}) are now recognized as emerging through a distinct T cell differentiation pathway independently of Th subset lineages and are instrumental in inducing germinal centre formation and promoting a range of B cell functions (King *et al* 2008; Crotty 2011; Cannons *et al* 2013). Bcl6, a transcriptional repressor, is the master regulator of these cells in mice and humans, and other key markers include CXCR5, programmed cell death protein-1 (PD-1) and inducible T cell costimulator (ICOS). They provide survival signals to germinal centre B cells, induce them to proliferate and hypermutate and mature into plasma cells, through interactions with surface molecules and secretion of IL-4 and IL-21 (Reinhardt *et al* 2009; King *et al* 2010; Crotty 2011). IL-21 provides a critical signal for the differentiation of B cells into plasma cells, and for protection against secondary challenge infection with *H. polygyrus* (King *et al* 2010). IL-21 plays a key role in the development of a protective Th2 immune response in helminth infections including *S. mansoni*, *N. brasiliensis* and *H. polygyrus* (Pesce *et al* 2006; Fröhlich *et al* 2007). T_{FH} are also the major IL-4-producers in B cell follicles of MLN after *H. polygyrus* (King and Mohrs 2009) and *N. brasiliensis* infections (Reinhardt *et al* 2009) and $ICOS^{-/-}$ mice have a defect in IL-4 producing T_{FH} in the MLN 14 days after *H. polygyrus* infection (Redpath *et al* 2013).

2.3 Dendritic cells

Dendritic cells (DCs) are the predominant innate APC which prime T cell responses, and are required in particular to prime Th2 responses against helminths (Banchereau *et al* 2000; Kapsenberg 2003; MacDonald and Maizels 2008; Paul and Zhu 2010; Phythian-Adams *et al* 2010). In order to prime a T cell response, the DC presents antigen on MHCII to be bound by the TCR (signal 1) and provides costimulation through expression of CD40, CD80 and CD86 (signal 2) (Caux *et al* 1994; Banchereau *et al* 2000; MacDonald *et al* 2002). However, whether a Th1 or Th2 response will result depends on the polarising cytokine environment (signal 3) (Kapsenberg 2003) and the strength of peptide binding by the TCR (Constant *et al* 1995). Low-level expression of MHCII, costimulatory molecules or low antigen concentration could all result in a weak TCR signal strength, which promotes Th2

responses (Constant and Bottomly 1997; Paul 2010). Weak TCR stimulation induces expression of the transcription factor GATA3, and early IL-2 and IL-4 production (Yamane *et al* 2005), with subsequent intracellular signalling events that promote Th2 cytokine production.

The recognition of Th2-inducing stimuli, such as helminth products and allergens, by an array of cell-surface pattern-recognition receptors (PRRs), induces DCs to make cytokines and chemokines that shape the direction of the immune response (Kapsenberg 2003; Perrigoue *et al* 2008; Everts *et al* 2010; Krishnaswamy *et al* 2013). As well as the traditional model of DC activation by ligation of PRRs, other mechanisms have recently been proposed that do not primarily involve these receptors (Pulendran and Artis 2012). For example, the enzymatic activity of certain allergens, and helminth molecules that resemble them (Bielory *et al* 2013), can activate DCs through protease-activated receptors (PARs), although the mechanisms involved are unclear (Pulendran and Artis 2012; Ramachandran *et al* 2012).

Relatively few specific helminth molecules have been identified that activate DCs (Hewitson *et al* 2009). The first such molecule was ES-62, a glycoprotein from the filarial nematode *Acanthocheilonema viteae,* which induced IL-4 production from bone marrow-derived DCs in culture (Whelan *et al* 2000). Omega-1, a glycosylated RNase in *Schistosoma* soluble egg antigen (SEA) is a potent inducer of Th2 responses through conditioning of DC (Everts *et al* 2009; Everts *et al* 2012). Furthermore, DCs pulsed with whole ES from *N. brasiliensis* (NES) can prime Th2 responses *in vivo* after transfer to naïve recipients (Balic *et al* 2004).

Helminth products have also been widely documented as modulators of DC function, and this may be a key way in which these parasites suppress immune responses to themselves, and bystander antigens/coinfections, *in vivo* (Segura *et al* 2007; Carvalho *et al* 2009; Terrazas *et al* 2010c; White and Artavanis-Tsakonas 2012). To this end, treatment of bone marrow-derived DCs with *H. polygyrus* ES (HES) impairs activation by the bacterial TLR9 ligand CpG (Segura *et al* 2007). Notably, treatment with *H. polygyrus* adult worm homogenate (AWH) did not have the same effect. Furthermore, addition of HES to DCs co-cultured with OT-II OVA-specific $CD4^+$ T cells resulted in a significant reduction of both Th1 and Th2 cytokines upon

OVA stimulation (Segura *et al* 2007). These findings suggest that *H. polygyrus* actively secretes a broadly suppressive factor for DCs, rather than selectively biassing the T cell response towards Th1 or Th2. SEA from *S. mansoni* can also suppress cytokine secretion, costimulatory molecule and MHCII expression on LPS-pulsed DCs (Kane *et al* 2004).

A mechanism by which helminth products modulate DCs to suppress immune responses is by directing them to specifically promote regulatory T cell responses. As examples, ES products from *Echinococcus multilocularis* (Nono *et al* 2012), *T. spiralis* (Aranzamendi *et al* 2012) and *Taenia crassiceps* (Terrazas *et al* 2011) all induce tolerogenic DCs which secrete IL-10 and induce $Foxp3^+$ Tregs *in vitro*. Furthermore, *H. polygyrus* has been shown to induce tolerogenic DCs that can block colitogenic cytokines IFN-γ and IL-17 in the gut, and upon transfer, protect against spontaneous inflammatory bowel disease (IBD) in $Rag^{-/-}$ mice reconstituted with $Il10^{-/-}$ T cells (Blum *et al* 2012). Surprisingly, this mechanism was Treg-independent and did not involve IL-10 or TGF-β.

In *H. polygyrus* infection settings, $CD11c^+$ DCs isolated from MLN show reduced expression of CD40, CD86 and MHCII, evoking lower Th1 cytokine expression, and containing a subset with a $CD11c^{low}$ $CD45RB^{mid}$ phenotype which promoted $Foxp3^+$ Treg differentiation via high production of IL-10 (Li *et al* 2011b; Smith *et al* 2011). The same reduced expression of costimulatory molecules and increased DC IL-10 production from DCs in the MLN of infected mice was seen in an earlier study in which phenotyping of DC subsets relied on CD80 and CD86 expression levels (Balic *et al* 2009). A CD11c.DTR mouse has been developed that co-expresses CD11c with the human diphtheria toxin receptor (DTR), so that upon administration of DTR, cells expressing CD11c are selectively depleted (Hochweller *et al* 2009). Although an incomplete depletion of $CD11c^+$ DCs from tissues is obtained using this system, an impaired Th2 cytokine response was observed upon infection with *S. mansoni* (Phythian-Adams *et al* 2010). It was observed that a specific subset of non-plasmacytoid $CD11c^{lo}$ DCs is spared DTR-mediated deletion, and these are tolerogenic in *H. polygyrus* infection, inducing Tregs and lower levels of antigen-specific $CD4^+$ T cell proliferation (Smith *et al* 2011). Finally, using mice with a Cre-

mediated constitutive, albeit incomplete, loss of all DC subsets impaired expulsion of *N. brasiliensis* was demonstrated (Ohnmacht *et al* 2009).

2.4 Alternatively-activated macrophages and their products

The alternative activation of macrophages is a characteristic feature of a helminth-elicited Th2 response and occurs in response to IL-4 and IL-13, in contrast to the 'classical' activation pathway induced by IFNγ in response to bacterial and viral infections (Gordon 2003; Gordon and Martinez 2010). Although the nature of macrophage metabolism means that these polarised activation states are reciprocally inhibitive of each other, macrophages have been shown to be extremely plastic and responsive to changing environmental stimuli, and are not of fixed terminal phenotypes (Hesse *et al* 2001; Mosser and Edwards 2008; Mylonas *et al* 2009; Menzies *et al* 2010).

Given that alternatively activated macrophages (AAMΦ) are induced by cytokines highly upregulated in helminth infection, many studies have now investigated them in the context of a variety of helminth models (Noel *et al* 2004; Kreider *et al* 2007; Reyes and Terrazas 2007; Jenkins and Allen 2010). Metabolic activity was one of the first tools used to distinguish between macrophages activated by Th2 cytokines - M2 or AAMΦ, versus Th1 – classically activated macrophages or M1 (CAMΦ). IL-4 and IL-13 induce the enzyme arginase 1 (Arg1) that competes with inducible nitric oxide synthase (iNOS) for the common substrate L-arginine (Munder *et al* 1998; Hesse *et al* 2001). Metabolism of L-arginine by Arg1 yields urea and ornithine. Orthinine is subsequently metabolised further into proline and polyamines, both of which are important in fibrosis and wound healing (Witte and Barbul 2003), widely proposed to be functions of AAMΦ and their products (Martin and Leibovich 2005; Wilson and Wynn 2009; Allen and Wynn 2011). Notably, in an *S. mansoni* egg injection model, in mice polarised to a Th2 response (IL-1-/IL-12 double-deficient mice), lung granuloma size and collagen deposition were greatly increased above levels in IL-4/IL-13-deficient Th1 polarised mice, and was associated with upregulation of AAMΦ-related genes (see below)(Sandler *et al* 2003).

During the early lung migratory stage of *N. brasiliensis* larvae, markers of AAMΦ are highly upregulated (Nair *et al* 2005; Reece *et al* 2006), and chronic or dysregulated production of profibrotic molecules by macrophages may result in long-term tissue damage in the lung of infected mice (Marsland *et al* 2008; Reece *et al* 2008; Laskin *et al* 2011). The precise role of AAMΦ in helminth-associated wound healing and/or fibrosis has not been well established, but as many helminths are tissue-migratory, it may be that these cells play a key role in limiting damage, bacterial translocation, and haemorrhage (Allen and Wynn 2011; Gause *et al* 2013). Compelling evidence for this theory came from *S. mansoni* infection in mice that lack the IL-4 receptor specifically in macrophages and neutrophils ($LysM^{Cre}IL\text{-}4R\alpha^{-/flox}$), and therefore cannot have any AAMΦ (Herbert *et al* 2004). All infected mice died, and this was associated with increased Th1 responses, liver and intestinal histopathology, impaired egg expulsion and sepsis.

The first analyses of gene expression in AAMΦ were on human IL-4 treated monocytes, and consistently showed upregulation of Arg 1, mannose receptor, MHCII, IL-4R, IL-1R and FcεRII (Dickensheets and Donnelly 1999; Hart *et al* 1999). A more comprehensive microarray analysis also exposed Ym-1 as one of the most higly upregulated genes in IL-4 and IL-13 treated murine macrophages, with expression dependent on the transcription factor STAT6 (Welch *et al* 2002). A further step was to show that macrophages elicited by live helminth (*B. malayi*) infection (called nematode-elicited macrophages or NeMΦ) also had abundant expression of Ym1, at over 10% of total mRNA from NeMΦ, as well as Arg 1, and another gene, RELM-α (also called FIZZ-1) (Loke *et al* 2002). These were all IL-4-dependent as upregulation did not occur in macrophages taken from $IL\text{-}4^{-/-}$ mice.

Research into the function of these abundantly-expressed molecules in both allergy and helminth-associated Th2 inflammation, and whether they have a direct role in immunity against parasites is ongoing (Nair *et al* 2006). Chronic infection of mice with *Trypansoma brucei brucei* results in a switch to Th2 cytokine production and alternative activation of macrophages (Namangala *et al* 2001), which display upregulation of Ym1 and RELM-α gene expression over macrophages from acute infection (Raes *et al* 2002). The same genes are also upregulated, and their proteins

detected, in tissues where parasites migrate and reside, as shown in peritoneal lavage (PL) from *B. malayi*-implanted mice, and tissues of mice infected with *L. sigmodontis*, *N. brasiliensis* (Nair *et al* 2005) and *T. spiralis* (Chang *et al* 2001).

More recently, comprehensive gene expression patterns have been analysed in IL-4-elicited AAMΦ using transcriptomic and proteomic approaches (Zhang *et al* 2010; Thomas *et al* 2012; Martinez *et al* 2013). The results from these analyses confirmed that genes already confirmed as markers of alternative activation in mice were highly upregulated in IL-4 treated murine macrophages, compared to IFN-γ- or IL-10-treated cells, along with a wide range of other cytokine-specific genes (Zhang *et al* 2010). Since there are no known homologues of Ym1 or RELM-α in humans, other signature markers are still being identified. Transglutaminase 2 (TG2) was proposed as an IL-4-induced gene common to mouse and human (Martinez *et al* 2013) which is highly upregulated, and involved in a range of diverse physiological processes including wound healing, fibrosis and apoptosis (Mehta *et al* 2010), all proposed functions for AAMΦ.

Ym1 (also called chitinase-3-like protein 3) expression is increased in several tissues associated with helminth infection and migration, as well as models of airway inflammation and fibrosis, and wound healing, leading to speculation that it might be a key player in the pro-fibrotic and wound repair functions of AAMΦ (Kawada *et al* 2007; Sutherland *et al* 2009; Daley *et al* 2010). Although Ym1 belongs to a family of chitinase-like proteins, which retain homology to plant and bacterial chitinases, no actual chitinase activity has been detected for native or recombinent Ym1 (Jin *et al* 1998; Chang *et al* 2001; Sutherland *et al* 2009). The presence of chitin and chitin synthase enzymes in certain lifecycle stages of some helminths, does suggest that if Ym1 retains chitin-binding activity, this could be a mechanism by which AAMΦs directly damage worms (Foster *et al* 2005). However, relevant in wound healing, Ym1 has been shown to bind heparin (Chang *et al* 2001), which is abundantly expressed on the extracellular matrix and is involved in cell adhesion and migration (Bernfield *et al* 1999; Tumova *et al* 2000). In addition, Ym1 is expressed in many situations where allergic inflammation occurs, and although originally identified as T-lymphocyte-derived eosinophil chemotactic factor (Owhashi *et al* 2000), this

function is disputed and it is not required for eosinophila (Welch *et al* 2002). Ym1 is also made by neutrophils (Harbord *et al* 2002), DCs (Cai *et al* 2009), mast cells (Lee *et al* 2005) and epithelial cells (Homer *et al* 2006), and is transiently expressed in several hematopoietic tissues during embryonic development (Hung *et al* 2002) and hence may serve multiple physiological roles *in vivo*.

RELM-α was originally identified as FIZZ1 (found in inflammatory zone 1) in bronchoalveolar lavage fluid (BALF) from inflamed lungs, and intestinal and lung epithelial cells (Holcomb *et al* 2000). However, it was subsequently named resistin-like molecule-α (RELM-α), belonging to a family of RELMs that are related to a hormone involved in glucose metabolism, made predominantly by fat cells (Steppan *et al* 2001). RELM-α is also expressed in white adipose tissue, heart and tongue (Steppan *et al* 2001). RELM-α is highly upregulated during helminth infection, with eosinophils as the primary producers in *S. mansoni*-associated lung and liver granulomas (Pesce *et al* 2009b), and is detected in the BALF and epithelial cells of egg-challenged mice (Nair *et al* 2009). In *B. malayi* infection, RELM-α was expressed by B cells and DCs in the draining lymph nodes, as well as AAMΦ in the PL (Loke *et al* 2002). RELM-α gene expression is also upregulated in the small intestine of *N. brasiliensis* and *T. spiralis*, and the colon of *T. muris*-infected mice, where protein was specifically localised to goblet cells with immunohistochemical staining (Wang *et al* 2005). Although an exact function for RELM-α has not been established, it has been proposed to have an imunoregulatory role, as it has been found to bind to macrophages and $CD4^+$ T cells in the lung of *S. mansoni* egg-challenged mice, and downregulate their cytokine expression (Nair *et al* 2009), and RELM-$\alpha^{-/-}$ mice (otherwise known as *Retnla*$^{-/-}$) display exacerbated granuloma-associated inflammation (Pesce *et al* 2009b). Expulsion of *N. brasiliensis* was also accelerated in these mice, accompanied by stronger Th2 responses, suggesting RELM-α is acting to downregulate potentially harmful over-exuberant fibrosis and Th2-mediated inflammation (Pesce *et al* 2009b).

To assess the importance of macrophages generally on the outcome of helminth infection, depletion using clodronate-loaded liposomes, which induce apoptosis of cells that ingest them, has provided a useful tool (van Rooijen and Hendrikx 2010).

For example, *N. brasiliensis* infection leads to Th2-dependent accumulation of macrophages with the molecular signatures of AAMΦ and high levels of gene expression of Arg1, Ym1 and RELM-α in the intestinal tissue (Zhao *et al* 2008). Upon administration of liposomes to *N. brasiliensis*–infected mice, resident and recruited F4/80^{+} macrophages are successfully depleted, although levels of Ym1 and RELM-α transcript are affected only marginally in gut tissue, illustrating that cell populations other than macrophages may be important producers of these products (Zhao *et al* 2008). However, Arg1 levels in the intestine of infected animals did decrease with liposome treatment, along with the magnitude of smooth muscle hypercontractility in the intestine. Macrophages were thereby shown to have a direct role in worm expulsion, as liposome-treated mice had significantly higher *N. brasiliensis* worm and egg burdens, despite unimpaired IL-4 or IL-13 production in the intestinal tissue (Zhao *et al* 2008).

AAMΦ are also a prominent feature of granulomatous inflammation around *H. polygyrus* in the intestinal submucosa (Morimoto *et al* 2004; Anthony *et al* 2006), especially in granulomas developed following a secondary challenge, which in immune-competent mice, results in an effective protective memory response (Patel *et al* 2009). However, mice treated with liposomes are unable to expel a secondary infection, and, as with *N. brasiliensis*, this is not accompanied by a failure to make a robust Th2 response (Anthony *et al* 2006). Furthermore, use of an Arg1 inhibitor, S-(2-boronoethyl)-1-cysteine (BEC) also abrogated protective responses and resulted in increased worm burdens (Anthony *et al* 2006), which correlated with decreased adult worm production of cytochrome oxidase, a molecule associated with worm stress (Takamiya *et al* 1996; Mei *et al* 1997), indicating a possible role for Arg1 in direct worm damage.

Another role for macrophages in helminth infection is the broader recruitment of other inflammatory cells to the infection site. In *N. brasiliensis* infection, AAMΦ were important in the recruitment of eosinophils to the peritoneal cavity, a site for significant inflammation in this model, even though the parasite does not migrate through it (Voehringer *et al* 2007b). Conversely, eosinophils have been shown to

sustain AAMΦ, in an IL-4- or IL-13-dependent manner, in adipose tissue, thus contributing to metabolic homeostasis (Wu *et al* 2011).

The anti-inflammatory effects of AAMΦ are also well documented, with macrophages from helminth-infected (*B. malayi*), animals, and those treated *in vitro* with Th2 cytokines being suppressive to the proliferation of T cells in an IL-4 dependent manner (Allen *et al* 1996; Loke *et al* 2000; Nair *et al* 2003; Mylonas *et al* 2009). Mechanisms of macrophage-elicited T cell suppression include depletion of L-arginine from the environment, as mentioned above (Pesce *et al* 2009a), production of the suppressive cytokines TGF-β and IL-10 (Osborne and Devaney 1999; Hesse *et al* 2004; Taylor *et al* 2006b), and through programmed death ligand (PD-L) interactions which have been shown to lead to T cell anergy in *S. mansoni* (Smith *et al* 2004) and *T. crassiceps* infections (Terrazas *et al* 2005). The classification of regulatory macrophages, or M2b cells, came to distinguish cells activated via regulatory cytokines, phagocytosis of apoptotic cells and immune complexes, rather than through Th2 cytokine stimulation or PRRs (Mosser and Edwards 2008).

Cytokines other than IL-4 and IL-13 have been shown to have a role in activating and regulating the effector functions of AAMΦ (Gordon and Martinez 2010). *Il21r*$^{-/-}$ mice display significantly reduced granulomatous inflammation and liver fibrosis in the *S. mansoni*-egg injection model, which is accompanied by reduced lung expression of Ym1, RELM-α, IL-4 and IL-13 (Pesce *et al* 2006). Th2 responses and expression of markers of AAMΦ were also reduced in these mice after *N. brasiliensis* infection compared to wild-type mice. *In vitro*, it was shown that IL-21 increases expression of IL-4R-α and IL-13R-α1 on macrophages, thus amplifying their IL-4 and IL-13-associated upregulation of Arg1 (Pesce *et al* 2009a).

A more recently identified mediator of AAMΦ recruitment is IL-33 which is well documented as a promoter of ILC2s (see section 2.11 below), and their production of type-2 cytokines which alternatively activate macrophages (Mirchandani *et al* 2012). Moreover, IL-33 has been shown to directly amplify the alternative activation of alveolar and bone marrow-derived macrophages by IL-4 and IL-13 *in vitro* (Kurowska-Stolarska *et al* 2009), to direct osteoclast precursors to an AAMΦ

phenotype (Zaiss *et al* 2011) and to polarise macrophages in the spleen and MLN of mice with experimental autoimmune encephalitis (EAE) to a protective AAMΦ phenotype (Jiang *et al* 2012). In *N. brasiliensis* infection, IL-33 promotes ILC2s, which in turn promotes accumulation of eosinophils and AAMΦ in visceral adipose tissue (Molofsky *et al* 2013).

Given the wide variety of tissue sites, migratory routes and required expulsion mechanisms for helminths, macrophages will have different roles in each model studied. However, there is compelling evidence that AAMΦ have the potential for important roles in direct worm killing, recruitment of other inflammatory effector cells, healing of helminth-associated tissue damage, prevention of over-exuberant and damaging responses, and directing the subsequent adaptive and memory responses to parasites.

2.5 Classically-activated macrophages

Although AAMΦ represent the primary activation phenotype of macrophages in helminth infection, CAMΦ are present in many helminth-associated inflammatory sites, and their actions may contribute significantly to anti-helminth immunity (Jenkins and Allen 2010). Nitric oxide (NO), the main product of L-arginine metabolism by iNOS, has been shown to directly harm certain worms, including *T. crassiceps* (Alonso-Trujillo *et al* 2007) and *Echinococcus granulosus* (Amri *et al* 2007). NO production is high in species of rodent naturally resistant to infection, and administration of a NOS inhibitor markedly increased *B. malayi* larval burden in these animals (Gupta *et al* 2004). It was proposed that NO synergises with superoxide in the peritoneal cavity of resistant animals, forming peroxynitrite radicals, which are highly toxic to *Brugia* larvae (Thomas *et al* 1997).

2.6 Mast cells

Mast cells are resident in peripheral tissues and at boundaries with the environment, and so are well placed to mount a quick response to allergens and invading

pathogens (Collington *et al* 2011). They are most well characterised as effectors of allergic responses, releasing histamine, TNF, a range of proteases, leukotrienes and prostaglandins during degranulation after the aggregation of FcεR1 by multi-valent allergen (Galli and Tsai 2010; Weller *et al* 2011). These mediators act to attract and activate further inflammatory cells, along with cytokines and chemokines released later in the response (Galli and Tsai 2010). Mast cell proteases particularly, are thought to negatively impact on epithelial barrier function, and increase permeability and fluid leakage, by altering tight junction proteins (Scudamore *et al* 1998; Jacob *et al* 2005; Groschwitz *et al* 2013). Mast cells have been shown to be potent Th2 cytokine producers (Nagarkar *et al* 2012), and in this way contribute to the promotion and maintenance of effective innate and adaptive anti-helminth immunity (Galli *et al* 2005; Galli and Tsai 2010; Urb and Sheppard 2012).

Using mast cell-deficient transgenic mice, the importance of mast cells in responses to helminth parasites, is being widely investigated. With regards to gastrointestinal helminths in particular, the roles of mast cells in epithelial barrier function are of particular interest. After administration of α-c-kit antibody, which prevents mastocytosis and the secretion of mast cell protease-1 (mMCP-1) in the small intestine, mice were unable to expel *T. spiralis* and this was due to a decrease in intestinal permeability (McDermott *et al* 2003). Furthermore, a specific deficiency in mMCP-1 had the same effect (Knight *et al* 2000; McDermott *et al* 2003). A breakdown in the epithelial barrier, and tight junctions, would lead to a major disruption of the niche of the parasite and contribute to its expulsion (Artis and Grencis 2008).

Mastocytosis and high levels of mast cell proteases correlate with the expulsion of other helminth parasites including *H. polygyrus* (Behnke *et al* 1993; Wahid *et al* 1994; Ben-Smith *et al* 2003), *N. brasiliensis* (Madden *et al* 1991; Lutzelschwab *et al* 1998) and *Strongyloides ratti* (Wilkes *et al* 2007). Most *in vivo* studies on mast cells in helminth infection have involved the mast cell deficient mice $Kit^{W}/Kit^{W\text{-}v}$, which have mutations in the gene that encodes the tyrosine kinase receptor c-kit. When these mice were infected with *H. polygyrus*, worm fecundity, but not adult worm number was increased, a phenomenon that was reversed upon reconstitution with mast cells (Hashimoto *et al* 2009b), indicating that mast cells play a minor

protective role in this system. However, a recent study showed that $Kit^{W}/Kit^{W\text{-}v}$ mice, and another mast cell-deficient strain $Kit^{W\text{-}sh}$, have significantly reduced Th2 responses and protective immunity to *H. polygyrus*, failing to limit egg production and adult worm numbers compared to C57BL/6 controls (Hepworth *et al* 2012). The reason for the discrepancy is not clear.

Of note is that c-kit-based deletion of cell types may not only be mast cell-specific, as the more recently described ILCs, cells important to the instigation of a Th2 response, have also been shown to express c-kit (Moro *et al* 2010; Neill *et al* 2010; Saenz *et al* 2010b). Assessing the extent to which these cells are affected in $Kit^{W}/Kit^{W\text{-}v}$ and $Kit^{W\text{-}sh}$ mice will be vital in determining the exact roles of mast cells in helminth infection.

2.7 Basophils

Recently, basophils have been the focus of renewed interest with regards to helminth-associated Th2 immunity, as both initiators and effectors of the response (Karasuyama *et al* 2011; Leon-Cabrera and Flisser 2012; Voehringer 2012; van Beek *et al* 2013; Voehringer 2013). Although constitutively present in blood circulation at low levels, their numbers increase dramatically in allergic inflammation and helminth infection settings. Basophilia is conspicuous in a number of experimental helminth infections, and basophils have been shown to be major IL-4 producers in the lung, liver and intestine of *N. brasiliensis*-infected mice (Min *et al* 2004; Voehringer *et al* 2004; Ohnmacht and Voehringer 2009; van Panhuys *et al* 2011), in the draining lymph node after food-pad injection of *S. mansoni* eggs (Perrigoue *et al* 2009) and in the spleen of *T. muris*-infected mice (Perrigoue *et al* 2009). As well as cytokine production, basophils act as APCs and in some situations preferentially induce Th2 responses over DCs with the same antigen (Yoshimoto *et al* 2009; Paul and Zhu 2010). The recruitment of basophils to allergic sites is well documented, and they have been shown to be important in the development of mast cell- and T cell-independent chronic allergic inflammation through the interaction of antigen-specific IgE and FcεR1 (Mukai *et al* 2005).

The depletion of basophils with the antibody MAR-1 impairs Th2 responses and *T. muris* worm expulsion (Perrigoue *et al* 2009), although this antibody binds to FcεR1, which is also expressed on mast cells, so the effects seen in this study cannot be attributed entirely to basophils. The same is true for αCD200R3, which has been used to show that basophils were necessary for the protective effects of an irradiated-larval vaccine to *L. sigmodontis* infection (Torrero *et al* 2013). Transgenic mice, that display constitutive and selective basophil depletion, have since been constructed (Ohnmacht *et al* 2010) and used to illustrate the importance of these cells for secondary immunity to challenge infections, rather than to primary infections, with *N. brasiliensis* (Ohnmacht *et al* 2010; Ohnmacht and Voehringer 2010). Although Th2 responses to primary infection were unaffected, a small, but significant, effect of basophil depletion on secondary immunity to *H. polygyrus* has also been shown, indicating that basophils contribute to this response, in concert with other mechanisms (Herbst *et al* 2012).

Studies with helminths, have contributed to the understanding of how basophil responses are induced. IL-3 has been shown to enhance antibody-mediated basophil activation and IL-4 production (Le Gros *et al* 1990a; Lantz *et al* 2008; Sullivan *et al* 2011), and in *H. polygyrus* infection, IL-3 is secreted by T-cells in an IL-4Rα-dependent manner (Herbst *et al* 2012). Interestingly, class-switched IgG1 and IgE antibodies were shown in the same study to promote basophil expansion in response to *H. polygyrus*, independently of IL-4Rα signalling (Herbst *et al* 2012). Furthermore, thymic stromal lymphopoietin (TSLP) (Siracusa *et al* 2011; Voehringer 2012) and IL-33 (Smithgall *et al* 2008; Suzukawa *et al* 2008; Blom *et al* 2011) have been shown to promote the activation and accumulation of basophils in helminth infections in both mice and humans. Indeed, when TSLP was neutralised with α-TSLP antibodies, *T. muris*-associated basophilia was severely reduced, independently of IL-3 (Giacomin *et al* 2012).

Through production of IL-4, basophils may promote anti-helminth responses by instructing T cells to differentiate into Th2 cytokine producers, although they are not necessary for this function in *N. brasiliensis* infection (van Panhuys *et al* 2011) or after injection of *S. mansoni* eggs (Sullivan *et al* 2011). Another study, however, showed that the interaction between $CD4^+$ T cells and basophils, via MHCII

interactions, promoted Th2 differentiation during *T. muris* infection (Perrigoue *et al* 2009). Basophils have also been shown to promote the alternative activation of macrophages and eosinophilia in response to *N. brasiliensis* (Ohnmacht and Voehringer 2009). In primary *L. sigmodontis* infection, basophils are not directly anti-parasitic, but do serve to amplify Th2 responses involving IgE production, T cell differentiation and eosinophila (Torrero *et al* 2010). It has also been shown that a failure to induce basophils in juvenile mice compared to adult mice contributes to their higher susceptibility to *N. brasiliensis* (Nel *et al* 2011). In addition to responding to TSLP, as above, basophils have also been shown to produce this cytokine, as well as IL-25 (Schneider *et al* 2010). These cytokines have been recognized as key players in the initiation of Th2 responses by promoting ILC2s (Paul and Zhu 2010; Oliphant *et al* 2011), an additional route by which cytokine production by basophils may mediate a protective Th2 response against helminths.

Basophils have been shown to have nonredundant roles in some settings, including in resistance to tick infestation in some animals, where basophils make up a large proportion of the skin cellular infiltrate at the feeding site (Karasuyama *et al* 2011). The secondary immune response to ticks was ablated in mast cell protease 8 (Mcpt8)-DTR mice that have a selective defect in basophils upon administration of diphtheria toxin (Wada *et al* 2010).

Although basophils have an undoubted role in the initiation of Th2 responses through the production of IL-4, and other cytokines, they are found to be responsible for immunity in only a very few helminth infection settings, suggesting that their functions are largely redundant and overlapping with other innate immune cell subsets.

2.8 Eosinophils

The association between eosinophils and helminth infection has been known for over 100 years (Brown 1898), with numerous reports of these cells killing worms *in vitro*, although there is much less evidence for an *in vivo* role (Klion and Nutman 2004).

Eosinophils have been shown to bind directly to *H. polygyrus* larvae *in vitro*, which significantly reduces their infectivity (Penttila *et al* 1983; Penttila *et al* 1984b), although no studies have described a role for these cells in the expulsion or killing of *H. polygyrus*. Eosinophils have been associated with the killing of the adult bovine parasite *Onchocerca ochengi* (Nfon *et al* 2006), and can attach to the cuticular surface of *N. brasiliensis*, releasing larvacidal mediators (Shin *et al* 2001). The same has been shown to occur with *S. mansoni* (Kassis *et al* 1979; McLaren and Ramalho-Pinto 1979; Olds and Mahmoud 1980).

Several systems for eosinophil depletion have been developed. Monoclonal antibodies to the eotaxin receptor, CCR3, can deplete eosinophils from the circulation, and significantly reduce eosinophilia in the lung and BALF following *N. brasiliensis* infection, although the effect on worm numbers or eggs was not reported in this study (Grimaldi *et al* 1999). The administration of αIL-5 antibody TRFK-5 resulted in the significant removal of eosinophils but resulted in no change in immunity to *N. brasiliensis* (Khan *et al* 1995), *H. polygyrus* (Urban *et al* 1991b) or to *T. spiralis* primary or secondary infection (Herndon and Kayes 1992), even though *in vitro*, eosinophils (or their products) were shown to be toxic to *T. sprialis* larvae (Bass and Szejda 1979; Buys *et al* 1981; Buys *et al* 1984; Gansmuller *et al* 1987). In certain settings however, an essential role for eosinophils in immunity has been established, as TRFK-5 treatment reduced eosinophilia and abolished the protective effects of irradiated larval vaccination to challenge infection with *L. sigmodontis* (Martin *et al* 2000). Ablation of eosinophils also led to an increase in worm survival in a model of *A. cantonensis* (Sasaki *et al* 1993).

IL-5 transgenic mice, which have constitutively high levels of IL-5 and eosinophils, displayed a lower *N. brasiliensis* intestinal worm burden, and the adoptive transfer of eosinophils from these mice to wild-type mice boosted their ability to clear the larvae from the lungs, suggesting that eosinophils play a role in anti-larval immunity at an early stage in migration (Shin *et al* 1997). Moreover, IL-$5^{-/-}$ mice, and mice in which the eosinophil-specific site in the GATA-1 promoter is deleted (ΔdblGATA), displayed impaired immunity to secondary *N. brasiliensis* infection, an effect seen on the numbers of larvae migrating to the lung, not from the lung to the intestine (Knott *et al* 2007). These findings together argue that eosinophils are most important in the

early-acting larval killing mechanism of tissue-migratory helminths, during the initial phases of infection and, particularly, reinfection.

Mechanisms of killing by eosinophils are not fully understood, although antibody and/or complement-mediated release of toxic granule proteins and reactive oxygen species have been proposed to be involved (Klion and Nutman 2004), and recently, cell degranulation has been demonstrated *in vitro* on contact with *Ascaris suum* infective larvae plus immune serum from pigs (Masure *et al* 2013). Specific toxic molecules made by eosinophils have been identified, such as major basic protein (MBP), which has been shown to be required for the eosinophil-dependent killing of *Strongyloides stercoralis in vivo*, although eosinophils are not absolutely required for immunity in this model (O'Connell *et al* 2011). Release of these toxic molecules can also result in tissue destruction and pathology (Klion and Nutman 2004).

Eosinophils are also a rich source of Th2 cytokines, as demonstrated by studies showing diminished Th2 responses in allergy models where eosinophils are ablated genetically and chemically (Mattes *et al* 2002; Walsh *et al* 2008; Spencer and Weller 2010), and are programmed early in their ontogeny, along with mast cells and basophils, for rapid IL-4 and IL-13 production (Gessner *et al* 2005). Furthermore, eosinophils are known to secrete Th2 cell-promoting chemokines, such as CCL7 (monocyte chemoattractant protein-3, MCP-3), CCL11 (eotaxin-1), CCL17 (thymus- and activation-regulated chemokine, TARC) and CCL24 (eotaxin-2)(Jacobsen *et al* 2008; Walsh *et al* 2008). Thus, as well as directly damaging helminths through the release of toxic mediators, eosinophils also promote and amplify protective Th2 responses.

2.9 Neutrophils

Neutrophils have been characterised as one of the earliest cell types recruited to sites of inflammation (Mollinedo *et al* 1999), and as producers of a wide variety of cytokines, chemokines and anti-microbial products (Cassatella *et al* 1997). However, neutrophils have not been studied to a great extent in the context of helminth infection. Indeed, neutrophils are the most numerous leukocyte, comprising over 50% of human blood cells (Makepeace *et al* 2012).

Neutrophils are prominent in the immediate cutaneous inflammatory response to invading *S. mansoni* cercariae (Incani and McLaren 1984), where they phagocytose larval ES material (Paveley *et al* 2009). As the first responders, neutrophils are thought to release chemokines to attract macrophages and DCs to the inflammatory site (Nathan 2006). This may also occur in *H. polygyrus* infection where they accumulate around the larvae as they invade the intestinal submucosa and are prominent in the primary, and to a lesser extent, secondary granuloma (Morimoto *et al* 2004; Anthony *et al* 2006; Patel *et al* 2009). They may have a direct detrimental effect on *H. polygyrus*, as pre-incubation of neutrophils with L3 larvae *in vitro* decreases their subsequent infectivity by 40-50%, an effect dependent on serum complement (Penttila *et al* 1983; Penttila *et al* 1984b). These researchers also showed that transfer of neutrophils enriched from day 4 *H. polygyrus*-infected mice to uninfected mice increased resistance to a secondary challenge infection (Penttila *et al* 1984a), and administration of a neutrophil-specific mAb completely abolished immunity to a challenge infection (Pentilla *et al* 1985).

Neutrophils have been shown to be protective in the particularly tissue-damaging migratory helminth *N. brasiliensis*, where $Gr1^+$ cell depletion resulted in early increased IFN-γ release, systemic bacterial infection and delayed worm expulsion (Pesce *et al* 2008).

A direct effect of myeloperoxidase from neutrophils was demonstrated in the killing of *S. stercoralis* tissue-migrating larvae, and a requirement for this protein in protection after secondary challenge was shown, although immunity was more effective when eosinophils or macrophages were also present (Galioto *et al* 2006; O'Connell *et al* 2011; Bonne-Annee *et al* 2013).

2.10 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogenous group of macrophage- and neutrophil-like cells which may be relatively immature but are able to exert immunosuppressive effects in environments such as tumour sites (Gabrilovich and Nagaraj 2009). Interestingly, MDSCs have been found to expand

in a number of helminth parasite infection settings and in response to parasite products (Marshall *et al* 2001; Terrazas *et al* 2001; Brys *et al* 2005; Gomez-Garcia *et al* 2005).

Although MDSCs are commonly defined in mice as $CD11b^{+}Gr1^{+}$ cells that lack expression of markers of mature myeloid cells, two major subsets have been characterised: monocytic MDSCs, expressing the cell surface marker Ly6C, and granulocytic, expressing Ly6G (Peranzoni *et al* 2010; Youn and Gabrilovich 2010). Both of these markers are bound by the α-Gr1 antibody RB6-8C5 used commonly in flow cytometry for identifying neutrophils, cells which are very hard to distinguish from granulocytic MDSCs without evaluating their suppressive potential (Fleming *et al* 1993; Brandau *et al* 2013; Lee *et al* 2013). However, studies have also been published that identify immunosuppressive populations of neutrophils (Arnardottir *et al* 2012; Pillay *et al* 2012), and some researchers have proposed that neutrophils and granulocytic MDSCs may be within the same heterogeneous family of cells, sharing similar morphological and functional traits (Pillay *et al* 2013). Furthermore, monocytic MDSCs are characterized in a similar way to inflammatory monocytes in their expression of Ly6C (Sunderkotter *et al* 2004).

The two subsets differ in the mechanisms used to suppress immune responses. Granulocytic MDSCs release reactive oxygen species (ROS), including peroxinitrite, which can cause post-translational modifications to TCRs, rendering $CD8^{+}$ T cells unresponsive to antigen (Nagaraj *et al* 2007; Gabrilovich and Nagaraj 2009; Youn and Gabrilovich 2010). Monocytic MDSCs use the two enzymes iNOS and Arg1, which metabolise the substrate L-arginine into NO, and urea and L-orthinine respectively (Gabrilovich and Nagaraj 2009). The catabolism of L-arginine depletes it from the microenvironment, which suppresses T cell responses through pathways such as the downregulation of CD3 and CD8 expression, and NO evokes multiple intracellular effects including suppression of IL-2 receptor signaling pathways in T cells, and the induction of apoptosis (Ochoa *et al* 2001; Bronte *et al* 2003; Bronte and Zanovello 2005; Choi *et al* 2009). These mechanisms can contribute to a worsened outcome in cancer (Nagaraj *et al* 2007; Raber *et al* 2012), protozoal

parasite infections (Munder *et al* 2009; Cuervo *et al* 2011) and viral infections (Chen *et al* 2011).

In the context of helminths, few studies have shown a role of for MDSCs. Recently, one study highlighted the context-dependent induction of the different MDSC subsets, in that monocytic MDSCs were able to suppress anti-tumour responses whereas only the granulocytic subset was able to enhance clearance of *N. brasiliensis* (Saleem *et al* 2012). Both mechanisms were reliant on mast cells, and the upregulation of a Th2 response protective against helminths, but which exacerbated metastasis. It has been shown that diversion of the local immune response in a Th2 direction can alternatively activate monocytes and MDSCs (Yang *et al* 2013a), which leads to subverted tumour immunity (Sinha *et al* 2007) but improved resolution of inflammation and tissue damage (Egawa *et al* 2013; Saiwai *et al* 2013), both properties that would be beneficial in helminth infections. Indeed these suppressive cells are induced and alternatively activated in the peritoneal cavity of mice infected with *T. crassiceps* (Brys *et al* 2005).

2.11 Innate lymphoid cells, IL-25, IL-33 and TSLP

Recently, a number of studies have identified a population of lineage-negative cells that produce type 2 cytokines (particularly IL-5 and IL-13) in response to the cytokines IL-25, IL-33 and TSLP (Barlow and McKenzie 2011; Oliphant *et al* 2011; Spits and Cupedo 2012). Although several publications originally described and named these cells in slightly different ways (Moro *et al* 2010; Neill *et al* 2010; Price *et al* 2010; Saenz *et al* 2010b), a uniform nomenclature of type-2 innate lymphoid cells (ILC2s) has been proposed (Spits *et al* 2013).

ILC2s require the expression of the transcription factor GATA3 (Hoyler *et al* 2012) and retinoic acid-related orphan receptor-α (Halim *et al* 2012; Wong *et al* 2012), express a number of cell surface markers (including c-kit, ICOS, ST2 and SCA1) and are involved in the initiation of both mouse and human Th2 responses in allergy and helminth infection (Mjösberg *et al* 2012; Walker *et al* 2013). Indeed, studies with helminth infection models were instrumental in the discovery of this crucial cell

population. It was already clear that protective Th2 cytokines could be made from innate cells in *N. brasiliensis* infection, as *Rag*$^{-/-}$ mice that lack an adaptive immune system, could still expel the worm, but not if these mice were also deficient in IL-4 or IL-13 (Voehringer *et al* 2006). It was subsequently found that lineage negative innate cells were the source of IL-5 and IL-13 before the T cell response had been initiated in *N. brasiliensis* infection, and that they were dependent on IL-25 (Fallon 2006). *Il25*$^{-/-}$ and *Il17br*$^{-/-}$ mice (that lack the common receptor for IL-25 and IL-33) are both unable to expel *N. brasiliensis* to the same extent as wild-type mice, corresponding with impaired Th2 responses (Fallon 2006; Neill *et al* 2010), and adoptive transfer of ILC2s can reverse this phenotype (Neill *et al* 2010), illustrating the key role of these cells in the development of a protective Th2 response. To date, the role of ILC2s in the immune response against *H. polygyrus* has not been characterised.

IL-25, IL-33 and TSLP are predominantly epithelial cell-derived, and are released upon damage, allergen exposure and helminth infection (Saenz *et al* 2008; Paul and Zhu 2010). IL-25, a member of the IL-17 family, is also made by other cell types, including mast cells (Ikeda *et al* 2003), basophils and eosinophils (Wang *et al* 2007), macrophages (Kang *et al* 2005) and T cells (Fort *et al* 2001; Owyang *et al* 2006a). As well as inducing ILC2s (see above), IL-25 also contributes to the activation of Th2 cells (Angkasekwinai *et al* 2007; Wang *et al* 2007) and limits chronic mucosal inflammation (Owyang *et al* 2006a). IL-25-deficient mice display reduced immunity to *T. muris* and exogenous IL-25 administration boosted immunity in the genetically susceptible mouse strain AKR (Owyang *et al* 2006a). The same mice had impaired Th2, intestinal smooth muscle and epithelial cell responses, and could not expel *N. brasiliensis* parasites (Zhao *et al* 2010). Mice in which the signalling molecule Act1, which acts downstream of IL-17 and IL-25 receptor ligation, can be selectively targeted in certain cells, showed that IL-25 signalling in epithelial cells, and not macrophages or T cells, was needed for ILC2 induction, and subsequent Th2 mediated *N. brasiliensis* expulsion (Kang *et al* 2012). As the release of IL-25 and IL-33 from epithelial cells was also compromised in these mice, it was proposed that there is a positive feedback loop mediated by IL-25, an observation also noted in mice infected with *H. polygyrus* (Hepworth *et al* 2012). Although no studies have

directly addressed the role for ILC2s in *H. polygyrus* infection, it is interesting to note that upon administration of rIL-25 to wild-type C57BL/6 mice, expulsion of *H. polygyrus* adults and limitation of egg production from worms was boosted significantly (Hepworth *et al* 2012).

IL-33, a member of the IL-1 family, has wide-ranging effects on a number of innate cells, in mice and humans, including promoting the alternative activation of macrophages (Kurowska-Stolarska *et al* 2009), activating mast cells (Allakhverdi *et al* 2007) and basophils (Smithgall *et al* 2008; Suzukawa *et al* 2008) and inducing ILC2s (see above). IL-33 production is induced by both *T. muris* (Humphreys *et al* 2008) and *Strongyloides venezuelensis* (Yasuda *et al* 2012), while *Il-33*$^{-/-}$ mice fail to expel both primary and secondary infections with *N. brasiliensis* (Hung *et al* 2013), a defect accompanied by a lack of IL-13-producing ILC2s and subsequent reduced innate and adaptive Th2 responses. Moreover, administration of rIL-33 early in *T. muris* infection confers resistance to normally susceptible AKR mice, mediated by an increased Th2 response to the worms (Owyang *et al* 2006b). IL-33 signals through the T1/ST2 receptor, and mice deficient in this make a suboptimal response to injected *S. mansoni* eggs, resulting in smaller granulomas due to reduced eosinophil infiltration, and a greatly reduced Th2 response (Townsend *et al* 2000b). However, *T1/ST2*$^{-/-}$ mice responded normally to *N. brasiliensis* infection (Hoshino *et al* 1999).

Trefoil factor 2 (TFF2) is a molecule involved in epithelial cell repair, which induces IL-33 production by epithelial cells in response to damage caused by *N. brasiliensis* (Wills-Karp *et al* 2012). *Tff2*$^{-/-}$ mice did not display elevated epithelial IL-33 levels in response to *N. brasiliensis,* compared to wild type mice, and had reduced Th2 responses and delayed worm expulsion (Wills-Karp *et al* 2012). The role of TFF2 in other helminth infections has yet to be established, although it is highly upregulated in the abomasal mucosa of sheep bred to be resistant to the helminth *Haemonchus contortus* shortly after a challenge infection (Nagaraj *et al* 2012).

Thymic stromal lymphopoietin (TSLP) is a key mediator of Th2-inflammation, and has been well characterized in models of skin and airway allergy (Al-Shami *et al* 2005; Jessup *et al* 2008; Siracusa *et al* 2011). In humans, TSLP expression in the bronchial mucosa correlates with severe asthma and chronic obstructive pulmonary

disease (Ying *et al* 2008), and polymorphisms in the TSLP promoter are associated increased risk of severe asthma (Harada *et al* 2011). It is thought to maintain both $CD4^+$ T cell, B cell and DC homeostasis, activate DCs to promote the survival of self-reactive T cells for selection as Tregs (Ziegler and Liu 2006; Ziegler and Artis 2010), and instruct DCs to skew T cells to a Type 2 phenotype (Soumelis *et al* 2002; Zaph *et al* 2007). *Tslpr*$^{-/-}$ or *Tpte2*$^{-/-}$ mice, which cannot signal through the receptor for TSLP, are unable to mount normal Th2 responses to inhaled antigen (Al-Shami *et al* 2005), and when infected with *T. muris* are skewed towards a Th1 response making them more susceptible to the worm (Zaph *et al* 2007; Massacand *et al* 2009; Taylor *et al* 2009a). Indeed, even in steady-state conditions, DCs isolated from these mice have elevated expression of IL-12/23p40 over DCs from wild-type mice (Zaph *et al* 2007). However, *Tslpr*$^{-/-}$ mice did make strong Th2 responses to *N. brasiliensis* and *H. polygyrus*, which were both expelled at the same rate as in wild-type mice (Massacand *et al* 2009). The explanation for the difference in necessity for TSLPR signalling in these helminth models was illustrated by the ability of excretory-secretory (ES) products from *H. polygyrus* and *N. brasiliensis* to mimic the role of TSLP, in downregulating the Th1 response from DCs, thus allowing protective Th2 response to develop. ES products from *T. muris* were unable to do this (Massacand *et al* 2009).

TSLP promotes basophilia as part of Th2-mediated inflammation (Siracusa *et al* 2011) and in experimental *T. spiralis* infection, it was shown that the early basophil response is critically dependent on TSLP, and this was necessary for a robust and protective Th2 response (Giacomin *et al* 2012). A deficiency in TSLPR signalling led to decreased eosinophilia and smaller granuloma size in a primary *S. mansoni* egg injection model, and less fibrosis early in infection with the parasite, that was accompanied by less pro-fibrotic IL-13-producing T cells in the liver (Ramalingam *et al* 2009). However, as infection progressed to chronicity, the levels of Th1 and Th2 cytokines produced were comparable between wild-type and transgenic mice.

2.12 Intestinal smooth muscle and epithelial cells

Among the range of IL-4, IL-5 and IL-13-mediated mechanisms shown to be important in the killing and expulsion of adult parasites from the gut is enhanced smooth muscle contractility in the small intestine, as shown in several helminth models including *T. spiralis* (Vallance *et al* 1997; Vallance *et al* 1999), *S. mansoni* (Marillier *et al* 2010) and *N. brasiliensis* (Zhao *et al* 2008). Although Th2- and Stat6-dependent smooth muscle contractility in the intestine is also enhanced after *H. polygyrus* infection (Zhao *et al* 2003; Au Yeung *et al* 2005; Shea-Donohue *et al* 2010), whether this is necessary for the elimination of parasites in this model is yet to be uncovered.

As well as acting on smooth muscle, Th2 cytokines can have effects on epithelial cell function and structure of the intestinal tissue itself, altering the intestinal environment such that it becomes hostile to helminth worms. *H. polygyrus* induces epithelial changes including increased permeability and chloride ion secretion in response to stimulation, and decreased glucose absorption (Shea-Donohue *et al* 2001), functions which are in part IL-4 and Stat6-dependent (Madden *et al* 2004). As *H. polygyrus* attaches to villi protruding into the lumen of the gut (Bansemir and Sukhdeo 1994), increased efflux and changes to epithelial cell absorption could interfere with their ability to attach and feed. Similar alterations are also observed in *N. brasiliensis* and *T. spiralis* infections (Madden *et al* 2004; Morimoto *et al* 2009). Using both *T. muris* and *T. spiralis* antigen preparations on *in vitro* epithelial cell lines, it has further been shown that intestinal epithelial cells (IELs) can be directly activated to engage NF-κB signalling pathways and induce expression of cytokines including IL-1β and IL-8, and molecules such as MHCII (Li *et al* 1998; Artis and Grencis 2008). Furthermore, the importance of epithelial cells in directing the correct immune response to helminths was demonstrated using *T. muris* infection in mice lacking IKK-β (a subunit of a molecule responsible for NF-κB activation), specifically in IELs (Zaph *et al* 2007). Infected mice failed to make a robust parasite-specific $CD4^+$ T cell response and are unable to eradicate the infection. Recently it has become clear that cytokines made by epithelial cells, such as IL-25, IL-33 and TSLP, are vital in inducing ILC2s which in turn direct and mediate Th2 responses to both helminths, and allergens (see section 2.11 above)(Saenz *et al* 2008).

2.13 Goblet cells and their products

Within the intestinal epithelial layer are specialised goblet cells that secrete innate defence proteins as well as large quantities of mucins, the key components of mucus, which forms a thick protective layer over the gut epithelium (Specian and Oliver 1991). Indeed, the mucus layer has been shown to be a key mechanism by which adult *N. brasiliensis* are trapped and excluded from infected rats (Miller *et al* 1981). Goblet cell hyperplasia is a dominant feature of the local response to intestinal helminths (Khan and Collins 2004), and is dependent on signalling induced by Th2 cytokines – mice lacking Stat6 had reduced numbers of goblet cells upon infection with *T. spiralis*, and had impaired expulsion of the worms from the gut (Khan *et al* 2001). During *H. polygyrus* infection, T- and B cell-deficient mice (SCID or nude mice) display compromised goblet cell hyperplasia and impaired worm expulsion (Hashimoto *et al* 2009a).

Individual components of intestinal mucus have also been investigated, such as mucins, which are biochemically altered by host immune mechanisms upon *N. brasiliensis* infection (Ishikawa *et al* 1993; Tsubokawa *et al* 2012). Expression of the secretory and the membrane-bound intestinal mucins, Muc-2 and Muc-3, is upregulated after *H. polygyrus, N. brasiliensis, T. muris and T. spiralis* infections, although whether they have a direct role in anti-worm immunity has yet to be established (Shekels *et al* 2001; Datta *et al* 2005; Kawai *et al* 2007; Hashimoto *et al* 2009a; Inagaki-Ohara *et al* 2011). However, Muc2-deficient mice displayed delayed expulsion of *T. muris* (Hasnain *et al* 2010). Muc5ac, a mucin not normally associated with the intestine, is highly upregulated in the cecum of *T. muris*-resistant BALB/c mice, and Muc5ac-deficiency rendered mice highly susceptible to this parasite, *N. brasiliensis* and *T. spiralis*, phenotypes that were not accompanied by reduced Th2 responses, or reduced expression of other key goblet cell mediators (see below) (Hasnain *et al* 2010; Hasnain *et al* 2011). Reduced ATP levels in worms exposed *ex vivo* to Muc5ac suggested that it might be having a direct effect on worm viability, but the exact mechanism by which this may be occurring has yet to be elucidated.

RELM-β (FIZZ-2) is a goblet cell-specific effector molecule, made in response to IL-4 and IL-13, in both humans and mice (He *et al* 2003; Artis *et al* 2004). It is important for normal intestinal epithelial barrier function and regulation of susceptibility to inflammation (Hogan *et al* 2006). Expression of RELM-β mRNA is very significantly induced in the gut upon infection with *N. brasiliensis*, *T. spiralis* and *T. muris*, to levels much higher than mucin and other effector molecule genes, and peak expression coincides with the height of Th2 cytokine production and worm expulsion (Artis *et al* 2004; Kawai *et al* 2007). RELM-β binds directly to structures on the integument of *T. muris* and *S. stercoralis in vitro,* impairing chemotaxis (Artis *et al* 2004), while in *H. polygyrus*, it impairs feeding, reducing both ATP and protein content of adult worms (Herbert *et al* 2009). Pre-treatment of *H. polygyrus* larvae with RELM-β, inhibits both fecundity and survival of adult worms in the mouse, and *Relmb*$^{-/-}$ mice cannot expel *H. polygyrus* or *N. brasiliensis* as quickly as wild-type C57BL/6 mice (Herbert *et al* 2009).

Goblet cells also secrete small peptide mediators from the TFF family, which serve critical functions in maintaining mucosal barrier function and integrity (Podolsky 1997; Buda *et al* 2012). Upon injury, TFF family members are released quickly to mediate rapid repair of epithelial cell layers, which may occur, at least with TFF2, via signalling through CXCR4, the proposed receptor (Dubeykovskaya *et al* 2009). High TFF2 expression is seen in allergic lungs of mice and humans, and in Th2-mediated inflammation after *N. brasiliensis* migration through the lung tissue (Wills-Karp *et al* 2012). *Tff2*$^{-/-}$ mice have delayed expulsion of adult *N. brasiliensis* worms and impaired control of egg production, and these correlate with lower early Th2 cytokine levels and IL-33 production, which is important for anti-*N. brasiliensis* immunity (Wills-Karp *et al* 2012). TFF2 was also found to be highly upregulated in the gut of sheep resistant to *H. contortus.* when compared to susceptible hosts (Nagaraj *et al* 2012).

Gene expression profiling of intestinal tissue after *N. brasiliensis* (Voehringer *et al* 2007a), *T. spiralis* (Pemberton *et al* 2004b) and *T. muris* infection (Datta *et al* 2005) in mice, and *T circumcincta* (Athanasiadou *et al* 2008) and *H. contortus* (Rowe *et al* 2009) in sheep, identified intelectin genes to be highly upregulated in immune

subjects over more susceptible hosts. Notably, intelectin-2 was found to be highly upregulated in the Paneth and goblet cells of the small intestine of resistant BALB/c mice after infection with *T. spiralis,* and the gene for this lectin is completely absent from the genome of the more susceptible C57BL/10 mouse strain (Pemberton *et al* 2004a). Intelectins are a family of lectins that can bind galactose residues on bacterial cell walls (Tsuji *et al* 2001; Tsuji *et al* 2007) and so could contribute to anti-helminth immunity by biochemically altering other mucus glycoproteins, or bind directly to parasite structures to prevent feeding or migration, in the way of RELM-β (Nair *et al* 2006). A further molecular component is angiogenin-4 (Ang4), a bacterially-induced anti-microbial peptide, made by Paneth cells in the small intestine, and goblet cells in the large intestine (Hooper *et al* 2003; Forman *et al* 2012). Expression of Ang4 appears in the colon earlier in mice resistant to *T. muris* than more susceptible strains, and is dependent on IL-13 (D'Elia *et al* 2009b; Forman *et al* 2012). The relevance of this mediator in other helminth models has not been characterised.

2.14 Type-2 granulomas

H. polygyrus provokes the formation of characteristic, macroscopic Th2-mediated inflammatory granulomas on the gut wall (Anthony *et al* 2007; Patel *et al* 2009). In both primary and secondary responses, a rapid accumulation of cells infiltrates the space around larvae invading the intestinal muscularis layer, at around day 4 post-infection (slightly later in primary infection), beginning with $Gr1^+$ $CD11b^+$ cells adjacent to the larvae (Morimoto *et al* 2004). A band of IL-4-expressing $CD4^+$ T cells then accumulate, and $F4/80^+$ macrophages, bearing IL-4Rα and CD206 (mannose receptor), indicative of alternative activation, are a predominant feature of the lamina propria and granuloma architecture (Anthony *et al* 2006). Some eosinophils and DCs also accumulate but not to the same extent as macrophages. Caution must be taken however, as our studies have shown that granuloma numbers vary between strains of mice in response to *H. polygyrus*, and whether the composition of the granuloma is also different has yet to be established (K. Filbey, in review, 2013). Also, most studies on granuloma composition have been undertaken

in secondary, challenge infection settings, and the importance of the primary granuloma is still to be uncovered.

In the latter stages of infection, cell necrosis and collagen deposition in the granuloma is observed (Cywińska *et al* 2004; Anthony *et al* 2006), illustrating the Arg-1 dependent proline production of AAMΦ that contributes to collagen (Wynn 2004; Allen and Wynn 2011). Clodronate-mediated depletion of macrophages, and administration of an arginase inhibitor, both abrogated secondary immunity to *H. polygyrus*, illustrating the key role for AAMΦ in this model (Anthony *et al* 2006).

Type-2 granulomas are also formed in the lung, intestine and bladder around tissue-invading eggs of *Schistosoma* parasites, which are mainly comprised of $CD4^+$ T cells, macrophages and eosinophils, and have a collagen-rich fibrotic structure (Pearce and MacDonald 2002). They provide a protective function in the trapping of eggs to prevent further migration and tissue damage, but the fibrotic plaques formed after granuloma resolution can be damaging to organ function and sometimes lethal (Pearce and MacDonald 2002).

2.15 Antibodies and B cells

As described above, protection against helminths is dependent on a strong Th2 response, which exerts itself in a variety of ways. The typical antibody isotypes associated with a type-2 response are IgG1 and IgE, which are induced primarily by IL-4 (Finkelman *et al* 1990). B cells, as well as producing antibodies as part of the humoral response, can present antigen, produce cytokines and express toll-like receptors and co-stimulatory molecules that promote, amplify and regulate the T cell response (Harris *et al* 2000; Gray *et al* 2007; Lund 2008; Alugupalli and Abraham 2009). Subsets of B cells have been described, similar to T cells, in the array of cytokines they produce, and which arm of the immune response they amplify and interact with (Harris *et al* 2000). Thus Be-2 cells produce IL-2, IL-4, IL-6 and IL-13 when stimulated with a Th2 stimulus, thereby further promoting protective Th2 responses, and Bregs (see below) can make IL-10 and TGF-β to regulate potentially damaging immune responses (Lund 2008; Lund and Randall 2010).

The importance of effector B cells in anti-helminth immunity, through mechanisms other than antibody production, has been specifically investigated (Harris and Gause 2011). Although a primary infection with *H. polygyrus* in B cell-deficient mice (JHD, $JH^{-/-}$ or μMT) results in similar worm and egg counts to wild-type mice, secondary immunity is dependent on the presence of B cells (McCoy *et al* 2008; Wojciechowski *et al* 2009; Liu *et al* 2010b). By constructing mixed-bone marrow chimaeras, it was found that lethally-irradiated mice reconstituted with bone marrow containing B cells unable to make antibody could still make a robust memory Th2 response to *H. polygyrus*, indicating an antibody-independent mechanism was involved (Wojciechowski *et al* 2009). In addition, reconstitution with bone marrow from $MHCII^{-/-}$, so that all B cells present were MHCII-deficient, resulted in highly susceptible mice compared to mice reconstituted with wild-type bone marrow (Wojciechowski *et al* 2009). The same was also true for mice reconstituted with bone marrow from $Il4r\alpha^{-/-}$ mice, showing that B cells primed through this receptor (Be-2 cells) are needed for protection. The *in vitro*, and *in vivo* production of IL-4 by B cells is also dependent on their expression of IL-4Rα and the presence of IL-4 itself (Harris *et al* 2005). Thus Be-2 cells regulate the Th2 memory response to *H. polygyrus* in an antibody-independent way.

Most investigators have focused on the specific role of antibody in mediating protection against *H. polygyrus*, for example studying mice with targeted deficiencies within the B cell compartment. Using selective isotype knockout mice given a secondary *H. polygyrus* infection, it was found that IgG1 is the major class-switched isotype leading to protection (McCoy *et al* 2008). Indeed, it has been long known that the humoral response against *H. polygyrus* is dominated by IgG1, and that serum fractions with highest parasite-specific IgG1 activity afford greater protection when transferred to an infected animal (Pritchard *et al* 1983). Transfer of secondary immune serum from a wild-type donor to another wild-type, or a B-cell deficient mouse, can significantly reduce the number of adult worms left in the intestine after a challenge infection (Harris *et al* 2006; McCoy *et al* 2008; Liu *et al* 2010b). However, the transfer of serum from secondary infection in mice that could not make *H. polygyrus*-specific antibodies did not confer protection. Furthermore, $AID^{-/-}$ (also known as *Aicda*) mice, that cannot undergo somatic hypermutation or

class switching, are not protected against secondary infection, indicating that parasite-specific, class-switched antibody is necessary for a protective memory response to this parasite (McCoy *et al* 2008; Wojciechowski *et al* 2009). In the strains studies these protective specificities only arise after multiple infections. A further feature is that primary *H. polygyrus* infection elicits a very large increase in non-specific serum IgG1 levels (hypergammaglobulinemia) (Prowse *et al* 1978; Chapman *et al* 1979; Pritchard *et al* 1983; McCoy *et al* 2008), but transfer of this serum is not protective against subsequent infection (Williams and Behnke 1983; McCoy *et al* 2008).

A similar role for B cells is seen in *S. stercoralis* infection, with μMT mice protected against a primary but not a secondary infection (Herbert *et al* 2002). μMT mice are also susceptible to *T. muris,* corresponding with a defective Th2 response. This phenotype, as in *H. polygyrus*, can be reversed by transfer of parasite-specific IgG1 from resistant mice (Blackwell and Else 2001), as can the susceptility of μMT mice to *B. malayi* (Gray and Lawrence 2002). However, both primary and secondary immunity to *N. brasiliensis* are uncompromised by a lack of B cells, and a robust Th2 response is made in the MLN in both settings (Liu *et al* 2010b).

Varying antibody isotype requirements are also seen in different helminth models. IgE is often cited as an important helminth-induced antibody isotype, being induced by IL-4 (Finkelman *et al* 1990). However, although there is a large increase in levels of IgE during many model helminth infections, few have displayed a requirement for IgE in protection (Zakroff *et al* 1989; Watanabe *et al* 2005; Erb 2007). For example, although *H. polygyrus* infection induces large increases in IgE levels (Katona *et al* 1991) $IgE^{-/-}$ mice have no defect in expelling this parasite (McCoy *et al* 2008). However, IgE is important in both *S. mansoni* and *T. spiralis* infections, during which, IgE-deficient mice have elevated larval and adult worm numbers compared to wild-type mice (King *et al* 1997; Gurish *et al* 2004). The primary mechanism of IgE-mediated immunity is activation of innate cells through ligation of the FcεR, and so its importance in parasite models is likely to reflect the requirement for mast cell, basophil and eosinophils in their expulsion (Raghavan and Bjorkman 1996; Watanabe *et al* 2005).

IgA is the primary antibody isotype involved in mucosal immunity (Macpherson *et al* 2008; Strugnell and Wijburg 2010). Although most Th2 associated cytokines are involved to some degree in induction of class-switching to IgA, TGF-β is the main cytokine known to be involved – mice deficient in the TGF-β receptor (TGFβRII) are almost completely deficient in IgA (Cazac and Roes 2000). Germ-free mice also have a profound deficiency in mucosal IgA production (Macpherson *et al* 2000) and it takes commensal colonisation of the neonatal intestine to induce localised IgA-secreting plasma cells (Macpherson *et al* 2008). Most IgA in the gut is made by plasma cells in the PP, which is then exported through the epithelial layer, having polymerised and bound to the pIgR (Brandtzaeg and Johansen 2005; Strugnell and Wijburg 2010). Faster increases and higher levels of intestinal and serum IgA correlate with greater resistance of different mouse strains to primary *H. polygyrus* infection (Molinari *et al* 1978; Ben-Smith *et al* 1999). *IgA*$^{-/-}$ mice display a small, but significant reduction in protective immunity after secondary infection with *H. polygyrus*, and IgA-deficiency in dogs does not prevent the expulsion of *S. stercoralis* (Mansfield and Schad 1992), illustrating that in some helminth models this isotype is not required for protection.

Differences in the requirement for B cells and antibodies in models of helminth infection illustrate the varying cellular and molecular mechanisms needed for protection, as described above. How antibodies contribute to worm killing or expulsion is not fully established. Antibodies have been shown to directly bind to a number of helminth larvae *in vitro* (Mitchell *et al* 1979) (Robinson *et al* 1997; McVay *et al* 1998), and larvae have also displayed antibody-dependent growth stunting (Ey 1988; Liu *et al* 2010b). Furthermore, serum from dogs treated with *Ancylostoma*-secreted protein (ASP) was able to inhibit migration of hookworm larvae *in vitro* to a greater extent than naïve serum (Bethony *et al* 2005), indicating that antigen-specific antibodies can bind to secreted products which contribute to worm migration and feeding. The production of monoclonal antibodies to parasite products, and more global BCR repertoire sequencing techniques, could represent a more directed approach to finding protective specificities and possible vaccine candidates.

2.16 Immunoregulatory cells and cytokines

Tregs are essential during an immune response in protection against immune mediated pathology whilst still allowing the response to act strongly enough to clear the pathogen (Belkaid and Tarbell 2009), and without them (in the Foxp3 mutant 'scurfy' mouse, and in humans with IPEX syndrome), autoimmune diseases lead to an early death (Lahl *et al* 2007; van der Vliet and Nieuwenhuis 2007). Foxp3 is used as the key marker of natural Tregs (which emerge from the thymus to limit self-reactive T cells in the periphery) and is also expressed by some induced Tregs - $CD4^+$ T cells that gain suppressive function upon antigen stimulation alongside exposure to IL-10, TGF-β and retinoic acid (Mucida *et al* 2007; Curotto de Lafaille and Lafaille 2009). In the absence of Foxp3, Tregs acquire effector T cell functions (Williams and Rudensky 2007) and the forced expression of Foxp3 confers suppressor function to $CD4^+$ $CD25^+$ T cells (Fontenot *et al* 2003), illustrating the importance of this transcription factor in Treg function.

Tregs are now recognised as a major cell subset induced by worms, and while first isolated and cloned in the context of human filarial helminth infection (Doetze *et al* 2000; Satoguina *et al* 2002), have since been found to be a major cell subset induced by worms in mouse models of filarial (McSorley *et al* 2008; Taylor *et al* 2009b), trematode (Taylor *et al* 2006a; Turner *et al* 2011) and cestode (Mejri *et al* 2011; Hernandez *et al* 2013) helminths. In *H. polygyrus* infection, they are induced early in infection in the MLN and spleen, with numbers peaking at day 28-post-primary infection (Finney *et al* 2007; Rausch *et al* 2008). Activation markers, such as CD103, CTLA-4 and ICOS, have been shown to be upregulated on Tregs following helminth infection and play key roles in their function (Finney *et al* 2007; Redpath *et al* 2013). Depletion of Tregs using various methods, can lead to the clearance of parasites, indicating their beneficial status to the survival of helminths *in vivo* (Taylor *et al* 2005; D'Elia *et al* 2009a).

H. polygyrus infection also induces $CD8^+$ Tregs in the lamina propria of the small intestine, which can inhibit T cell proliferation *in vitro* in an IL-10 and TGF-β independent manner (Metwali *et al* 2006; Setiawan *et al* 2007).

Regulatory B cells (Bregs) have also been described, that produce IL-10 and TGF-β, and can dampen potentially harmful immune responses (Mizoguchi and Bhan 2006; Lund and Randall 2010). These cells have been shown to mediate the protection from anaphylaxis given by *S. mansoni* infection (Mangan *et al* 2004), and those induced by *H. polygyrus* infection can be transferred to protect against allergic airway inflammation and EAE in recipient mice (Wilson *et al* 2010). IL-10-producing B cells have also been found in multiple sclerosis patients naturally infected with helminths, and these cells can downregulate proliferative responses of T cells *in vitro* (Correale *et al* 2008).

IL-10 and TGF-β have long been known to be key mediators of regulation during parasitic infections (Sher *et al* 1992; Allen and Maizels 2011). IL-10 is made by a wide variety of cells and acts to inhibit both innate and adaptive immune responses, often being found essential in the prevention of immunopathology associated with infections (Couper *et al* 2008). IL-10 is released from PBMCs of healthy and *Schistosoma haematobium* infected people upon stimulation with worm homogenate and SEA, and antigen-specific IFN-γ production is enhanced when anti-IL-10 is added to the culture (King *et al* 1996). In mouse models, IL-10 has been shown to be crucial in preventing *S. mansoni* induced liver pathology, by downregulating inflammatory Th1 cytokine responses (Wynn *et al* 1998), and in expulsion of *T. muris* (Schopf *et al* 2002). IL-10 deficient mice displayed significantly higher morbidity and mortality, when infected with *T. muris*, than wild-type C57BL/6 and IL-4 deficient mice, and this was dependent on the presence of IL-12, suggesting that without the regulation of IL-10, the Th1 response is detrimental to the host (Schopf *et al* 2002). A similar outcome was observed after *T. spiralis* infection in *Il10*$^{-/-}$ mice (Helmby and Grencis 2003).

Evidence from a range of sources suggests that the role of IL-10 in *H. polygyrus* infection is less critical to control pathology than in other models (Maizels *et al* 2011). Although *H. polygyrus* infected animals display high levels of antigen-specific IL-10 production, and transfer of total MLNC from infected to uninfected animals conferred protection from allergic airway inflammation, MLNC from IL-10-deficient animals had the same effect, indicating that IL-10 is not the primary

immunomodulator in this system (Wilson *et al* 2005). IL-10 deficient mice develop spontaneous severe colitis, which is ameliorated by infection with *H. polygyrus* and accompanied by a reduction in intestinal IL-12 and IFN-γ responses (Elliott *et al* 2004). Furthermore, the protection against type-1 diabetes in NOD mice by *H. polygyrus* is IL-10 independent (Liu *et al* 2009), except in a Th2-deficient environment, such as in NOD IL4$^{-/-}$ mice or with blockade of IL-10 alongside IL-4 with neutralising antibodies (Mishra *et al* 2013).

In terms of innate immunity, IL-10 downregulates IgE-dependent stimulation of basophils in chronic infections with *L. sigmodontis* and *S. mansoni* (Larson *et al* 2012b), and infiltration of basophils into the peritoneal cavity of *B. malayi*-infected mice (Simons *et al* 2010). Chronic helminth infection is also associated with decreased basophil responsiveness in humans (Larson *et al* 2012a).

TGF-β controls immune responses through regulation of activation, survival and chemotaxis of numerous innate and adaptive cells, and is important in the prevention of immunopathology and in wound healing (Li *et al* 2006). Levels of plasma TGF-β are high throughout infection with *H. polygyrus,* and are significantly reduced upon anti-helminthic drug treatment (Su *et al* 2005). Anti-TGF-β administration prevented adult worm expulsion in BALB/c mice at 6 weeks post infection, when this strain has usually cleared the majority of worms from the intestine (Doligalska *et al* 2006) and application of the TGF-β signalling inhibitor SB413526 in infected mice prolongs infection (Grainger *et al* 2010). Although TGF-β$^{-/-}$ mice are not viable, a genetic construct in which T cells express a dominant-negative form of the TGF-β receptor (TGF-βRII^{dn}) allows survival of mice into adulthood (Gorelik and Flavell 2000). These mice eventually develop spontaneous inflammatory bowel disease, which infection with *H. polygyrus* cannot cure (Ince *et al* 2009), indicating an essential role for this cytokine in immunomodulation by the parasite. TGF-βRII^{dn} mice are also fully susceptible to *H. polygyrus* infection (Ince *et al* 2009; Reynolds and Maizels 2012), with significantly lower CD103 expression on regulatory T cells and an increase in levels of IFN- γ released from CD4^{+} and CD8^{+} T cells (Reynolds and Maizels 2012). TGF-βRII^{dn} mice have reduced survival of natural regulatory T

cells (nTregs), and exogenous TGF-β is a potent inducer of Foxp3 expression and suppressive function in $CD4^+$ T cells (induced Tregs) (Li and Flavell 2008).

It is clear that ES products from helminths are also potent inducers of Tregs and regulatory cytokines (Hewitson *et al* 2009), in some cases mimicking the pathways of IL-10 and TGF-β described above. Thus, naïve $CD4^+$ splenocytes upregulate Foxp3 expression upon incubation with HES, in a manner dependent on the TGF-β signalling pathway (Grainger *et al* 2010), whilst ES products from *Schistosoma* cercariae are able to induce host IL-10 production in the same way (Jenkins *et al* 2005).

2.17 The host microbiome

In recent years, the importance of the host microbiome has been demonstrated in the development of a fully functional immune system, in modulation of the immune response to pathogens and in development of autoimmune diseases such as IBD (Garrett *et al* 2010; Sekirov *et al* 2010; Hooper *et al* 2012). Findings from the Human Microbiome Project show an abundance and diversity of bacteria in several different habitats in the human body, which vary dramatically between individuals (Consortium 2012). In the gut in particular, the composition of the microbiome changes along the length of the gut, which is the most highly colonised site in the body, due its high surface area and availability of nutrients (Sekirov *et al* 2010). Bacterial concentration increases from small to large intestine, and different populations of bacteria inhabit the deeper, mucus-rich layers compared to the layers proximal to the lumen, indicating that a range of microbiome-induced effects will be seen in different areas of the intestinal tissue (Swidsinski *et al* 2007; Larsson *et al* 2012).

In the study of the microbiome mouse models, such as germ-free or specific pathogen-free mice, to investigate the importance and impact of bacterial populations on the development, homeostasis and health of the immune system have proved invaluable (Yi and Li 2012). For example, the presence of discrete populations of bacteria along the gut, polarise naïve T cells towards particular subset fates, which

could have an impact on the local immune response and therefore the response to parasites colonising that specific area of the intestine (Ivanov *et al* 2009; Atarashi *et al* 2011; Atarashi *et al* 2013).

Relatively few investigators have directly studied the interaction of the gut microbiome and gastrointestinal helminths (Berrilli *et al* 2012). Early reports indicated that *H. polygyrus* could not infect germ-free mice as well as conventionally colonised animals, suggesting that bacteria may promote helminth infection (Wescott 1968; Weinstein *et al* 1969; Chang and Wescott 1972). However, *H. polygyrus* infection has been shown to shift the composition of intestinal bacteria from that in naïve mice (Walk *et al* 2010; Reynolds *et al* 2013), although it remains to be seen whether this shift acts to promote longevity of parasite infection, and if so, if HES contains an element that promotes specific bacteria, or any changes that reflect a more general alteration in the immunological or ecological environment of the intestine.

3. HELMINTH EXCRETORY-SECRETORY PRODUCTS

Secretions of larval helminths were first investigated with regards to developmental (egg hatching, larval exsheathment, sheath or cyst formation) or invasive (lytic and enzymatic) functions (Stirewalt 1963). The term "excretory-secretory" (ES) has been used for many years to describe all products which are released from living helminths, including those actively shed from the parasite surface and those released from specialised internal organs (Lightowlers and Rickard 1988). Radio-labelled molecules from the surface of worms (eg. *S. mansoni* (Kusel *et al* 1975) and *T. spiralis* (Philipp *et al* 1980)), molecules associated with the gut of worms (eg. gut associated circulating anodic proteoglycan from *S. mansoni* (Nash and Deelder 1985)) and those found in discrete organs of the worm (eg. acetylcholinesterase from excretory glands of *N. brasilensis* (Ogilvie *et al* 1973) and *Stephanurus dentatus* (Rhoads 1981)) have been identified in ES material from *in vitro* culture and were found to be highly immunogenic.

Antibodies and complement, and therefore cells, can bind to the parasite surface and carry out effector functions – antibodies specific to larval lifecycle stages of both *T. spiralis* and *N. brasiliensis* were isolated from infected rats and found to facilitate the binding of peritoneal exudate cells to the larval surface (Mackenzie *et al* 1978), and sera taken from elephantiasis patients was found to promote adhesion of peripheral blood cells to *Wuchereria bancrofti* microfilariae *in vitro* (Subrahmanyam *et al* 1978). Both IgG and complement protein C3, in the serum of infected cats, were found to be involved in the adherence of eosinophils and neutrophils to *B. pahangi* microfilariae *in vitro* (Johnson *et al* 1981).

3.1 Identification of secreted products

The first attempts at identification of these immune system targets were with surface radiolabeling of *T. spiralis* using ^{125}I-labelled iodine, which revealed a lifecycle stage and sex-specific profile of expression of protein antigens on the larval surface (Philipp *et al* 1980; Adams *et al* 1988). In addition, some surface antigen

preparations were found to be protective against challenge infection (Grencis *et al* 1986), demonstrating the immunological significance of these molecules.

Early characterisation of ES products, rather than purely surface molecules, included radiolabelling and separation of antigens on SDS-PAGE gels, and immunoblotting, to assess their molecular weight and immunogenicity (Maizels *et al* 1984; Parkhouse *et al* 1985; Kennedy and Qureshi 1986). Fractionation and immunoprecipitation allowed further biochemical and functional analysis (Zimmerman and Clark 1986; Adams *et al* 1987). Screening of parasite cDNA libraries with immune sera allowed the identification of immunogenic genes, as with *Onchocerca volvulus* (Bradley *et al* 1991), *S. haematobium* (Renganathan *et al* 1993) and *Brugia* species (Selkirk *et al* 1987), which could then be characterized biochemically to assess their function, and searched against other published genes.

A wider approach was later taken with the sequencing of random clones from cDNA libraries to generate Expressed Sequence Tags (ESTs), which massively increased the information available, and new genes described (Blaxter *et al* 1999; Parkinson *et al* 2003). Analysis of ESTs from *N. brasiliensis* showed that a significant proportion of cDNAs possess predicted signal sequences and can therefore presumably code for secreted proteins in particular (Harcus *et al* 2004). In addition, a large number of sequences with the signal peptide were novel, in that they had little homology to other known helminth sequences (Harcus *et al* 2004). Similar projects have been undertaken for other helminth parasites including *B. malayi* (Blaxter *et al* 1996) and *S. mansoni* (Franco *et al* 1995). Others went further and identified ES proteins from 39 different species of parasitic nematodes (human, animal and plant parasites) using ESTs (Nagaraj *et al* 2008) and a Helminth Secretome Database (HSD) based on ESTs has been set up online (Garg and Ranganathan 2012).

Bioinformatic approaches to identifying helminth ES proteins have been very fruitful, however, using predicted signal sequences to define secreted products, may underestimate and miss key components (Hewitson *et al* 2009). Some studies have shown that a proportion of ES proteins (Cass *et al* 2007; Bennuru *et al* 2009) and specific molecules known to be in ES material, for instance, Macrophage Migration Inhibitory Factor (MIF) (Swope *et al* 1998), do not possess signal sequences.

Therefore, proteomics approaches have been adopted to establish a more realistic picture of what ES material contains.

Proteomic analyses of ES (secretomes) from a number of helminths have been published, often comparing life-cycle stages, and highlighting major protein families with multiple variants (Hewitson *et al* 2009). Several secretomes of *B. malayi* have been analysed and matched against the published genome (Ghedin *et al* 2007; Hewitson *et al* 2008; Moreno and Geary 2008; Bennuru *et al* 2009). These studies used a combination of SDS-PAGE electrophoresis and microcapillary reversed-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) to compare lifecycle stages, and male and female adults. The majority of proteins found at each stage in the lifecycle are specific to that stage (Bennuru *et al* 2009), as are proteins in males versus females (Moreno and Geary 2008). In addition, a large subset of proteins is seen to be enriched in ES, compared to whole worm extract, including many known immunomodulators (Hewitson *et al* 2008). Notably, one of the most abundant proteins found to be secreted by adult worms was triosephosphate isomerase (TPI), an enzyme involved in glycolysis. Variants of this enzyme have been found in other helminth parasites (Jiménez *et al* 2003), and have also been used as DNA-vaccine candidates against *Schistosoma* species (Zhu *et al* 2004; Da'dara *et al* 2008).

3.2 *H. polygyrus* ES (HES)

ES from *H. polygyrus* (HES) has been studied for many years, and, although methodology and the technology involved has improved, many intriguing effects of HES were discovered several decades ago (Maizels *et al* 2011). A study comparing the immunoprecipitation of antigens by immune serum, found that only one common immunogenic antigen (at 60,000 MW) was recognised in both worm cuticle and ES preparations by secondary serum (Adams *et al* 1987). A similarly sized antigen (of 66,000 MW) was found be specifically enriched in male ES over female ES, shown with radiolabelling and on silver-stained gels, and was immunoprecipitated with immune serum (Adams *et al* 1988). The same 60,000 MW antigen was found to be a

major glycoprotein of ES and the cuticle, and was successfully used to reduce the fecundity of *H. polygyrus* adults in an infection upon vaccination (Monroy *et al* 1989d).

Identification of enzymatic activity started to build a picture of the complex nature of HES, with proteases (Monroy *et al* 1989b), acetylcholinesterase (Lawrence and Pritchard 1993), and acid phosphatase (Martínez-Grueiro 2002) all described in both invasive tissue larval stages and adults. Others found direct evidence for immunomodulatory activity of HES - when fractionated by size, low molecular weight antigens were found to be suppressive of mitogen-stimulated lymphocyte proliferation (Monroy *et al* 1989c) and several immunomodulators were found to inhibit antibody production by splenocytes to KLH *in vitro* (Pritchard *et al* 1994; Telford *et al* 1998). HES has also been found to inhibit activation of DCs by TLR stimuli, leading to limited cytokine production and maturation marker expression (Segura *et al* 2007), and to downregulate IL-12p40 production from cultured DCs in response to LPS stimulation, independently of TLR signaling (Massacand *et al* 2009).

Proteomic analysis of HES from worms raised in resistant or susceptible mouse strains demonstrated the plasticity of the secretome of *H. polygyrus* – proteins overproduced in worms from the susceptible strain (C57BL/10) compared to those from SWR mice (fast responders) were notably globins, calreticulin and phosphatidylethanolamine-binding protein (Morgan *et al* 2006). Recombinant *H. polygyrus* calreticulin has since been found to be highly immunogenic and skews responses from $CD4^+$ T cells to Th2 *in vitro* (Rzepecka *et al* 2009). Two independent groups have undertaken extensive analysis of proteins in HES and more than 300 have been catalogued (Hewitson *et al* 2011b; Moreno *et al* 2011). These studies have highlighted several dominant families represented in HES including the enzymes mentioned above (proteases and acetylcholinesterases), other enzymes including apyrase and lysozyme, globins and vitellogenins (Hewitson *et al* 2011b; Moreno *et al* 2011).

The largest protein family represented in HES, and the most abundant, is the glycoprotein Venom Allergen-Like (VAL) family, with 25 variants (Hewitson *et al* 2011b), which are closely related to families found in ES from other helminths such

as the ASP family from *Ancylostoma caninum* (Mulvenna *et al* 2009). All belong to the sperm-coating protein (SCP)-like extracellular protein family (Cantacessi and Gasser 2012). There is no known function for VALs, but their expression is wide-spread among mammalian- and plant-parasitic nematodes (Gao *et al* 2001; Chalmers *et al* 2008). VALs are secreted across life cycle stages in *S. mansoni,* with some groups preferentially expressed only by invading or migrating cercariae, and some only by mature adults (Farias *et al* 2012). ASPs are expressed in different parts of the helminth structure, both on the cuticle and in the gut and secretory glands (Zhan *et al* 2003). There is immunomodulatory activity reported for some specific SCP family members including induction of neutrophil and monocyte migration (Bower *et al* 2008) and inhibition of platelet aggregation and adhesion (Del Valle *et al* 2003). We found VALs to be highly immunogenic, with dominant antibody responses being raised against VAL glycans during *H. polygyrus* infection (Hewitson *et al* 2011a).

Potential immunomodulatory proteins found in HES include MIF, cysteine protease inhibitors (CPI), C-type lectins and a variety of proteases (Hewitson *et al* 2011b; Moreno *et al* 2011). Homologues of MIF have been found in a number of helminth parasites (of which many have been demonstrated to possess its enzymatic tautomerase activity) including *B. malayi* and other filarial worms (Pastrana *et al* 1998; Falcone *et al* 2001; Prieto-Lafuente *et al* 2009), *S. ratti* (Younis *et al* 2011), *T. circumsincta* (Nisbet *et al* 2010), *Anisakis simplex* (Park *et al* 2009) and *T. spiralis* (Tan *et al* 2001), and indeed in protozoan (Jang *et al* 2011; Zhang *et al* 2011; Sun *et al* 2012) and ecto parasites (Wasala *et al* 2012). MIF has a range of diverse functions in the mammalian immune system (as discussed in chapter 3), and the homologues cloned from various parasites display a similar variety of functions. *B. malayi* MIF homologues have been shown to inhibit the random migration of human monocytes (Pastrana *et al* 1998; Tan *et al* 2001), and to contribute to the alternative activation of macrophages in a Th2 environment (Prieto-Lafuente *et al* 2009). It has also been shown to induce the release of pro-inflammatory cytokines from monocytes, including endogenous host MIF (Zang *et al* 2002) and to act as a chemoattractant for macrophages and eosinophils (Falcone *et al* 2001).

A CPI from *H. polygyrus* has recently been cloned and found to have immunomodulatory effects on developing DCs from bone marrow *in vitro* – costimulatory molecule expression and cytokine production are reduced and the ability to prime T cells is also hampered (Sun *et al* 2012). The C-type lectins released by *H. polygyrus* and *N. brasilensis* share sequence homology to mammalian immune receptors (the macrophage mannose receptor CD206 and the low affinity FcεR CD23), which may point to a diversionary role in the immune response towards these parasites (Harcus *et al* 2009). HES includes a wide array of proteases and the proteolytic activity of these are highest in early L3 larval stages suggesting a role for tissue migration and invasion (Lawrence and Pritchard 1993). These could potentially also serve to degrade host immune molecules that would otherwise signal and promote effector functions against the larvae.

3.3 Vaccination and helminth therapy

Knowledge of the immunomodulators secreted by parasitic helminths could be of great benefit in designing new interventions to clear existing and prevent further infections. The neutralisation of molecules that would otherwise divert immune responses away from the worm could unleash the full capacity of the immune system to eliminate parasites. Indeed, vaccination with recombinant proteins from parasitic helminths is already proving promising in animal models (Tendler *et al* 1995; Hotez *et al* 2003; Da'dara *et al* 2008; Pearson *et al* 2010; Babayan *et al* 2012). Most recently, a large field trial was undertaken on a human hookworm vaccine using the *Necator americanus* homologue of *Ancylostoma* Secreted Protein-2 (Na-ASP-2) (Schneider *et al* 2011).

The protective qualities of the presence of helminths on a range of immunopathological diseases (McKay 2006) has lead to a huge interest in using live worm infection, or isolated molecules from worms, as preventative and curative agents (Harnett and Harnett 2010; Osada and Kanazawa 2010). The data from models of disease in rodents are certainly promising. The tapeworm *Hymenolepis diminuta* is more effective at reducing symptoms of chemically-induced colitis in

mice, than dexamethasone (Melon *et al* 2010), and *S. mansoni* has been shown to reduce arthritis severity in a mouse model (Osada *et al* 2009). Whether attenuated parasites or parasite products will be effective as a live infection is unclear (Osada and Kanazawa 2010). However, it has been shown that the suppression of allergic airway inflammation by *H. polygyrus* in mice (Wilson *et al* 2005), can be replicated with HES alone (McSorley *et al* 2012). *H. polygyrus* has also been shown to bring about remission in mice with EAE through a switch in the cytokine profile of serum and cerebral spinal fluid (Donskow-Åysoniewska *et al* 2012), to block colitis through induction of tolerogenic DCs (Hang *et al* 2010; Blum *et al* 2012) and to reduce insulitis and type-1 diabetes (Liu *et al* 2009; Mishra *et al* 2013).

Both *Trichuris suis* and *N. americanus* have now been licensed for human trials and have been tested with varying degrees of success in patients with multiple sclerosis, IBD, celiac disease and allergic rhinitis (Khan and Fallon 2013). For example, *T. suis* ova given orally, six times over a twelve week period, were found to be safe and effective at improving the health of ulcerative colitis sufferers (Summers *et al* 2005). A small cohort of MS patients in Argentina who also became infected with various helminth species showed greatly reduced disease severity scores, fewer nervous system lesions and less IFN-γ and IL-12 producing PBMCs than uninfected MS sufferers (Correale and Farez 2011). Importantly, these improvements were completely reversed after anti-parasite treatment was administered (Correale and Farez 2011). Improvement of symptoms of human ulcerative colitis and idiopathic chronic diarrhoea in macaques has also been shown with concurrent *Trichuris trichuria* infection (Broadhurst *et al* 2010; Broadhurst *et al* 2012).

ES-62, a glycoprotein from the filarial worm *A. viteae,* has been shown to have a wide range of anti-inflammatory effects (Harnett *et al* 2010) including reduction of arthritis progression, both when administered before and after disease onset in mice (McInnes *et al* 2003). Other defined parasite-derived immunomodulators that have been documented in disease therapy include PAS-1 from *A. suum* which had a suppressive effect on allergic airway inflammation, and AvCystatin, a CPI from *A. viteae,* which reduced airway allergy to grass pollen extract (Danilowicz-Luebert *et al* 2012).

Further experimentation into the safety and effectiveness of both helminth and helminth-derived product therapy, and finding the precise cellular and molecular mechanisms behind the effects they have, are necessary before long-term treatment programs can be put in place (Tilp *et al* 2013). There are obvious concerns with using live worm infections to treat human diseases, but the effectiveness of attenuated worms, or helminth products, may be limited due to the fact that live worms can regulate their production of important molecules and fine-tune the localised immune environment in which they live in a manner that non-living molecules applied systemically may not be able to reproduce (Osada and Kanazawa 2010).

4. THESIS OBJECTIVES

Although there is a broad literature on anti-helminth immunity, studies with *H. polygyrus* in particular have often focussed on secondary infection settings, especially with regards to the roles of B cells and antibodies. Primary responses are important in the development of effective memory responses to subsequent infection and in the characterization of helminth antigens first recognized by the immune system. Therefore, a number of transgenic mice strains were given primary infections with *H. polygyrus* to measure the impact of defects in the B cell and antibody immune compartments on the ability to expel the worm and control parasite egg producation. Parasite-specific primary antibody responses were then mapped to components of HES with the aim of determining the nature of immunodominant antigens secreted by the parasite and to test the protective qualities of specific antibodies.

Secondly, a wide-ranging study of the immune responses to primary *H. polygyrus* infection in four mouse strains, with varying resistance to the parasite, was undertaken. Although adaptive immune responses are well characterised for this parasite, the role of macroscopic granulomas on the small intestine have not been evaluated, and innate componants of the response have been less widely studied. Therefore, levels of T cell-derived cytokines, T regulatory cell and antibody

responses were assessed, along with innate cell populations including macrophages, which are the dominant cell type in intestinal granulomas, and the newly described innate lymphoid cells, which have not previously been documented in *H. polygyrus* infection, with the aim of finding key immune parameters that correlate with an ability to expel the worm.

The cytokine MIF is a pleiotropic and widely expressed mediator that has not been studied in depth with regards to helminth infection and Th2 responses, although there is evidence that it can amplify innate type-2 responses in some settings, including the alternative activation of macrophages and the recruitment of eosinophils. As these are dominant cell types in the type-2 innate response to helminths, MIF was investigated in detail with relation to immunity to *H. polygyrus* and *N. brasiliensis* – parasites that have not previously been investigated with regards to this immune mediator. Primarily using infections in $MIF^{-/-}$ mice, the importance of MIF in key aspects of the immune response to, and the subsequent clearance of, both parasites, was uncovered. Specifically, innate lymphoid cells, which have not previously been linked to MIF, were investigated, along with the myeloid cell compartment, eosinophils and adaptive T and B cell responses, with the aim of assessing the role of MIF in primary immunity to both helminth parasites.

The immunomodulatory properties of helminths are widely documented although specific molecular mediators from *H. polygyrus* have not been fully characterised. HES has previously been shown to have TGF-β activity and this was further dissected by fractionation of proteins by size and charge. Finally, the TGF-β activity in isolated fractions was then investigated using a proteomic-based approach to create a shortlist of candidates for further investigation, with the hope that future recombinant versions of these candidates could be protective in disease models.

Materials and Methods

Mice, parasites and products

CBA, C57BL/6, BALB/c, and SJL mice, IL-4Rα$^{-/-}$ (BALB/c), IFN-γ$^{-/-}$ (C57BL/6), MIF$^{-/-}$ (BALB/c), MD4 (BALB/c), μMT (C57BL/6), CD40$^{-/-}$ (C57BL/6), and CD154$^{-/-}$ (C57BL/6) mice were bred in-house and housed in individually ventilated cages (IVCs) according to UK Home Office guidelines.

Infections employed 200 L3 larvae of *H. polygyrus* in 200 μl water by oral gavage, or 250 *N. brasiliensis* L3 larvae sub-cutaneously in 100 μl water. Parasite lifecycles were maintained as previously described in F1 mice or Sprague-Dawley rats (Camberis *et al* 2003). HES and NES were collected every 2-3 days from adult *H. polygyrus* and *N. brasiliensis* cultured in parasite media for up to 21 days, centrifuged at 12,000 rpm for 10 mins to remove eggs, and concentrated over a 3000 MW cut-off membrane in an Amicon Ultra Centrifugal Filter unit (Millipore). After approximately 1 L of media had been concentrated to 5 ml, the membrane was washed with 50 ml PBS and again concentrated to 5 ml, which was sterile-filtered through a syringe-tip 0.2 μm filter (Millipore) and frozen at -80°C. HEX was prepared by homogenizing adult *H. polygyrus* worms in ice-cold PBS, centrifuging at 13,000 rpm for 10 mins, sterile-filtering through a syringe-tip 0.2 μm filter and samples were frozen at -80°C.

Granuloma and adult worm counts were conducted after small intestines were removed and sliced longitudinally. 3-4 faecal pellets were weighed and dissolved in 2 ml dH_20. 2 ml of saturated salt solution (400 g NaCl in 1L dH_20) was added and eggs enumerated using a McMaster egg counting chamber. Egg counts are represented as eggs/g faecal material.

For secondary infections, mice were cleared of worms after 28 days infection with *H. polygyrus* with pyrantel embonate Strongid-P paste, given in 2 doses of 2.5 mg dissolved in 200 μl dH_20 given a day apart by oral gavage. After 2 weeks, mice were re-infected with 200 L3 larvae.

Media

cRPMI - RPMI 1640 medium (Gibco) containing 10% FCS, 100 U/ml penicillin, 100 µg/ml Streptomycin and 2 mM L-glutamine (all Sigma).

Parasite media – RPMI with 100 U/ml penicillin, 100 µg/ml Streptomycin, 2 mM L-glutamine and 1% sterile glucose (Sigma). For *H. polygyrus* only, 1 µg/ml of gentamycin (Sigma) is also added.

Lung media – Hanks Balanced Salt Solution (HBSS)(Sigma) with 100 U/ml penicillin, 1.8 mM $CaCl_2$ and 1 mM $MgCl_2$ (all Sigma).

Lung digest media – lung media (above) with 4 U/ml Liberase TL (Roche) and 160 U/ml DNase I (Sigma).

FACS buffer – 1x PBS containing 0.5% bovine serum albumin (BSA) (Sigma), and 0.05% sodium azide (Sigma).

ELISA carbonate buffer – 45 ml of 1M $NaHCO_3$ and 18 ml of $NaCO_3$ (both Sigma) made up 1 L with dH_20, pH 9.6.

Hybridoma media – RPMI with 20% Fetalclone 1 FCS (Hyclone), 100 U/ml penicillin, 100 µg/ml Streptomycin, 2 mM L-glutamine, 5 ml OPI media supplement (1 mM oxaloacetate, 0.45 mM pyruvate, 0.2 U/ml insulin)(Sigma) and 10 ml HAT media supplement (100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine)(Sigma).

D10/D2.5 media – DMEM (Sigma) with 10% FCS (D10) or 2.5% FCS (D2.5), 100 U/ml penicillin, 100 µg/ml Streptomycin and 2 mM L-glutamine.

Immobiline strip rehydration buffer – 7M urea, 2M thiurea, 4% CHAPs, 65 mM DTE, 0.8% IPG buffer pH 4-8 and bromophenol blue (trace), in dH_2O.

Immobiline strip equilibration buffer – 1 M Tris-HCl pH 6.8, 6M Urea, 60 ml glycerol, 2% SDS, bromophenol blue (trace) made up to 200 ml dH_2O.

Cell isolation and culture

Mesenteric lymph node (MLN) cell suspensions were prepared directly by passage through 70 μm nylon filters (BD) and placed in cRPMI. Peritoneal exudate cells were collected by washing the peritoneal cavity with 2 x 5 ml RPMI using a 23 gauge needle. Red blood cells were removed by adding 3 ml red blood cell (RBC) lysis buffer (Sigma) for 4 minutes, and washing with cRPMI.

Bronchoalveolar lavage (BAL) cells were harvested by washing lungs in 3 x 1 ml of 0.5% BSA in PBS. Samples were centrifuged at 12,000 rpm for 5 mins, with the first wash being centrifuged separately to collect fluid for ELISAs, whilst all cells were combined for further FACS analysis. Cells were digested from one lobe of the lung by disrupting by hand in 0.5 ml "lung media", on ice. 0.5 ml "lung digest media" was added and incubated for 25 mins in a shaking 37°C incubator. 100 μl 0.5M EDTA was added to stop the digestion, and total volume made up to 5 ml with cRPMI. Cells were pushed through a 70 μm strainer and RBC lysed as described.

Cells were then either stained for flow cytometry, or restimulated in cRPMI with 1 μg/ml HES or NES, 2 μg/ml αCD3 or medium alone for 72 hrs at 37°C, and cytokine production measured by ELISA.

MACS separation and FoxP3 induction

Splenocytes, having been treated with RBC lysis buffer as above, were magnetically labelled with CD4 (L3T4) microbeads (Miltenyi Biotec) as per the manufacturer's instructions. $CD4^+$ cells were separated using a MACS separator. $5x10^5$ $CD4^+$ cells were cultured for 72 hours, in 24-well plates, with 0.2 μg/ml αCD3, 1 μg/ml αCD28, 20 ng/ml IL-2, and either cRPMI, or varying dilutions of HES or TGF-β. Following incubation, cells were FACS stained for FoxP3, CD103, CD4, and CD25 as described.

Staining and flow cytometry

Cells were stained in 96-well round-bottomed plates. Prior to FACS antibody staining of cells, cells were washed in PBS and stained with LIVE/DEAD (Invitrogen) at a 1/1000 dilution in 100 μl PBS for 20 min at 4°C. Then, Fc receptors were blocked in 50 μl of FACS buffer containing 100 μg/ml of naïve rat IgG (Sigma) for 20 min at 4°C. Samples were then washed in 200 μl of FACS buffer and surface stained for 20 min in 20 μl of FACS buffer containing a combination of the following antibodies:

Marker	Colour	Clone no.	Manufacturer	Catalogue no.
INNATE LYMPHOID CELL STAINS				
CD3*	FITC	17A2	Biolegend	100204
CD8α*	FITC	53-6.7	Biolegend	100706
CD11b*	FITC	M1/70	Biolegend	101206
CD11c*	FITC	N418	Biolegend	117306
CD19*	FITC	6D5	Biolegend	115506
Gr1*	FITC	RB6-8C5	Biolegend	108406
F4/80*	FITC	BM8	Biolegend	123108
MHCII*	FITC	M5/114.15.2	Biolegend	107606
CD49b*	FITC	DX5	eBioscience	11-5971.85
CD4*	APC/Cy7	RM4-5	Biolegend	100526
* = lineage markers for negatively gating ILCs				
ICOS	PerCP e710	15F9	eBioscience	46-9940-82
IL-5	PE	TRFK5	eBioscience	12-7052-82
IL-13	eF660	eBio13A	eBioscience	50-7133-82
MYELOID CELL STAINS				
F4/80	PerCP-Cy5.5	BM8	Biolegend	123128
CD11b	Pacific Blue	M1/70	Biolegend	101224
CD11c	APC	N418	Biolegend	117310

SiglecF	PE	E50-2440	BD	552126
Ly6C	FITC	HK1.4	Biolegend	128006
Ly6G	APC/Cy7	1A8	Biolegend	127624
CD115	APC	AFS98	eBioscience	17-1152-82
Relm-α	226033 (R&D) and rabbit IgG AF647 labelling reagent kit (Invitrogen)			
Ym1	biotinylated goat α-mouse Chitinase 3-like 3 (R&D) and Streptavidin-PeCy7 (Biolegend)			
LYMPHOCYTE STAINS				
CD4	A700	RM4-5	Biolegend	100536
CD8	PerCP	53-6.7	Biolegend	100732
FoxP3	APC	FJK-16s	eBiosecience	17-5773-82
GATA-3	PeCy7	L50-823	BD Pharmingen	560405
CD103	Biotin/Streptavidin PerCP	M290	BD Pharmingen	557649
CD25	PE	PC61.5	eBioscience	12-0251-82
IL-4	PE	11B11	Biolegend	504104
IL-10	Pacific Blue	JES5-16E3	Biolegend	505020
IFN-γ	PeCy7	XMG-1.2	BD Bioscience	557649

To measure intracellular cytokines, cells were first stimulated for 4 hrs at 37°C in the presence of PMA (50 ng/ml), Ionomycin (1 μg/ml), and Brefeldin A (10 μg/ml) (all from Sigma). Following surface staining, cells were permeabilised for 30 min at 4°C in Cytofix/Cytoperm solution (BD), and then washed twice in 200 μl of Perm/Wash (BD). Cells were stained for intracellular cytokine expression in the same manner as for surface markers but substituting perm/wash for FACS buffer.

For Foxp3, GATA-3, RELM-α and Ym-1, samples were stained for surface markers after which cells were permeabilised for 12 hrs at 4°C in Fix/Perm solution (eBioscience Foxp3 staining set), and then washed twice in 200 µl of Perm/Wash (eBioscience Foxp3 staining set). Cells were stained in the same manner as for surface markers but substituting Perm/Wash for FACS buffer.

After staining cells were washed twice in 200 µl of FACS buffer before acquisition on the LSR II or Canto flow cytometer (BD Bioscience), and subsequently analysed using FlowJo (Tree Star).

Cytokine ELISAs

Cytokine levels were detected in culture supernatants by ELISA using monoclonal capture and biotinylated detection antibody pairs as follows, used at concentrations optimised previously:

	Capture Ab	**Conc.**	**Top standard concentration**	**Detection Ab**	**Conc.**	**Manufacturer**
IL-4	11B11 (made in-house)	2 µg/ml	8 ng/ml	BVD6-24G2	1 µg/ml	BD Pharmingen
IL-10	JES5-2A5	4 µg/ml	10 ng/ml	SXC-1	2 µg/ml	BD Pharmingen
IL-13	eBio13A	4 µg/ml	10 ng/ml	eBio1316H	0.5 µg/ml	eBioscience
IFN-γ	R46A2 (made in-house)	2 µg/ml	50 ng/ml	XMG1.2	1 µg/ml	BD Pharmingen

96-well ELISA plates (Nunc) were coated overnight at 4°C with capture antibody at the concentrations indicated above in 50 µl carbonate buffer. Plates were washed in Tris-buffered saline with 0.1% Tween 20 (Sigma) (TBSt) and blocked for 2 hr with 200 µl TBST with 10% FCS, at 37°C. Recombinant standards were prepared at the

stated concentrations in the same media as the samples, and added in doubling dilutions, in duplicate, including blank wells of media alone. Both samples and standards were added in 50 µl, and plates were incubated overnight at 4°C. Plates were washed in TBSt and detection antibody added, in TBST with 5% FCS, at the stated concentration, for 1 hr at 37°C. After washing in TBSt, streptavidin-alkaline phosphatase (Sigma) was added to wells in 50 µl TBST with 5% FCS at a 1/12500 dilution, for 45 mins at 37°C. Plates were washed in TBSt and distilled water, and 50 µl per well of *p*-nitrophenyl phosphate (pNPP, 1 mg/ml, Sigma) substrate was added, and plates incubated at room temperature in the dark. OD was measured at 405 nm on a Precision microplate reader (Molecular Devices) as the assay developed. Softmax Pro software was used to calculate the standard curve and concentrations of cytokines in samples were automatically calculated from this.

Gut and lung homogenate

Approx. 1 cm small intestine or 1 lobe of the lung were homogenised in 500 µl 1x lysis buffer (Cell Signalling Technology Inc) plus 5 µl phenylmethanesulfonyl fluoride solution (PMSF) (Sigma) using a TissueLyser (Qiagen). Samples were centrifuged at 12,000 rpm for 10 mins to remove debris and supernatants added to ELISAs, at a 1:10 dilution, to measure RELM-α, Ym1, IL-25 or IL-33 with antibody pairs described. Levels were normalised to protein content measured using a Bradford assay.

Protein	Capture antibody	Top standard	Detection antibody	Manufacturer
RELM- α	polyclonal rabbit anti-mouse RELM- α	50 ng/ml	biotinylated goat anti-mouse RELM-α	Peprotech 500-P214
Ym1	rat anti-mouse Chitinase 3-like 3	20 ng/ml	biotinylated goat anti-mouse Chitinase 3-like 3	R&D Duoset DY2446
IL-25	rat anti-mouse IL-17E	4 ng/ml	biotinylated goat anti-mouse IL-17E	R&D Duoset DY1399
IL-33	goat anti-mouse IL-33	2 ng/ml	biotinylated goat anti-mouse IL-33	R&D Duoset DY3626

Antibody ELISAs

Whole blood was clotted overnight at 4°C and then spun for 20 mins at 13,000 rpm to remove RBC. After blocking at 37°C with 10% BSA in carbonate buffer, serum was subsequently added in serial dilutions to ELISA plates coated with either 1 μg/ml HES, goat α-mouse Ig (Southern Biotech) at 1 μg/ml or anti-IgE (clone R35-72, BD Biosciences) at 1.5 μg/ml, in carbonate buffer. Antibody binding was detected using HRP-conjugated goat anti-mouse IgG1, IgG2a, IgA or IgE (Southern Biotech) and ABTS Peroxidase Substrate (KPL), and read at 405 nm. For HES-specific IgE ELISAs only, IgG was depleted from serum by incubating with Protein G agarose FastFlow beads (Millipore) overnight at 4°C, with rotation.

***In vivo* treatments**

Clodronate liposome treatment was conducted as described elsewhere (Anthony *et al* 2006; Jenkins *et al* 2011), administering 200 μl clodronate i.v. on days 0, 1, 3 and 6 of infection, with peripheral blood sampling at day 7. Control mice received PBS.

For anti-CD8 treatment mice were injected with 200 µg anti-CD8a clone YTS169 i.p. on days -1, 0, 2, 5, 7, 9, 12, 14, 16, 19, 21, 25 and 27, before experimental harvest at day 28. Control mice received rat IgG (Sigma) (protocol designed and carried out by Dr James Hewitson).

400 ng of recombinant IL-25 (R&D) in 200 µl PBS, or PBS alone, was administered i.p. on days 0, 1 and 2, and MLN harvested on day 3 for analysis of ILC induction (Neill *et al* 2010).

200 ng of recombinant IL-33 (R&D) in 50 µl PBS, or PBS alone, was given i.n. whilst mice were briefly sedated after inhalation with isofluorane, on days 0, 1 and 2. BAL and lung tissue for digestion were harvested on day 3 (adapted from (Bartemes *et al* 2012)).

10 µg of *Alternaria alternatus* antigen (Greer) in 50 µl PBS, or PBS alone, was administered i.n. whilst mice were briefly sedated after inhalation with isofluorane. Mice were sacrificed 1 hr or 48 hrs later for BAL and lung tissue harvest (adapted from (Bartemes *et al* 2012)).

50 ng of recombinant MIF (R&D) in 50 µl PBS, or PBS alone, was administered i.p. every other day, during either *H. polygyrus* or *N. brasiliensis* infection (adapted from (Park *et al* 2009)).

1 mg of MIF inhibitor, 4-IPP (Tocris Bioscience)(Winner *et al* 2008), in 50 µl DMSO, or DMSO alone, was administered i.p. every other day, during either *H. polygyrus* or *N. brasiliensis* infection (adapted from (Yaddanapudi *et al* 2013)).

Immunohistochemistry

Transverse sections were made from 2 cm of paraffin-embedded small intestine, at a thickness of 4 μm using a cryostat. Sections were directly stained with hemotoxylin and eosin or toluidine blue stain (for mast cells), or processed for immunostaining with α-Ym1 or α-MIF antibodies. Briefly, sections were deparaffinised by immersing slides in Histoclear (Brunel Microscopes Ltd) for 5 mins, and then hydrated through 100%, 95% and 70% ethanol successively. Antigen retrieval was carried out by immersing slides in citrate buffer (20 mM citric acid + 0.05% Tween 20 at pH6) warmed to 95°C for 20 mins. Slides were washed twice in 1x PBS, sections ringed with a wax pen and 200 μl block (1x PBS + 1% BSA, 2% normal rabbit serum, 0.1% Triton X-100 and 0.05% Tween 20) added for 30 mins at room temperature.

Rat α-mouse Ym1 (R&D) or rat IgG control (Sigma) was added at 25 μg/ml in block buffer, or rabbit α-MIF (Invitrogen) at 1:2000 dilution in block buffer, and left overnight at 4°C. Slides were immersed in 3% H_2O_2 for 10 mins at room temperature, and washed in PBS. Rabbit α-rat IgG (for Ym1) or goat α-rabbit (for MIF) conjugated to biotin (Vector Laboratories) at 5 μg/ml in PBS was added for 1 hour at room temperature, in the dark. Following 2 washes in PBS, several drops of ABC Vectastain (Vector Laboratories) were added and slides left for 30 mins at room temperature, in the dark. Slides were washed twice in PBS and DAB peroxidase solution (Vector Laboratories) was added for 5 mins (until a brown stain had developed).

With water washes in between, the following were added successively to counterstain the sections: Harris hemotoxylin solution (Sigma), acid alcohol (75% ethanol + 1% HCl) and Scott's Tap Water Substitute (ddH_2O + 42 mM $NaHCO_3$ and 167 mM $MgSO_4$). Slides were dehydrated through 75%, 95% and 100% ethanol and then Histoclear added for 5 mins. Coverslips were added with DPX mountant (Sigma) and slides were left to dry overnight, in the dark. Pictures were taken using a Leica DFC290 compound microscope and Leica Application Suite software.

RNA extraction and quantitative PCR

Approx. 0.5 cm of the uppermost part of the duodenum was placed into 1 ml of TRIzol (Invitrogen), and extracted according to the manufacturer's protocol. Briefly, tissue was first disrupted using a TissueLyser (Qiagen), then 200 μl chloroform was added and samples were centrifuged at 12,000 *g* for 15 min at 4°C. The upper aqueous layer was recovered and added to 500 μl of isopropanol, mixed, and stood at room temperature for 10 min. The sample was then centrifuged again at 12,000 *g* for 10 min at 4°C. Pelleted RNA was washed once in 70% ethanol, and allowed to air dry before being dissolved in 50 μl of DEPC-treated water; 15 μl RNA was treated with DNAse (DNAFree kit, Ambion), concentrations were determined using a Nanodrop 1000 (Thermo Scientific), and samples reverse-transcribed using 1-2 μg of RNA with M-MLV reverse transcriptase (Promega). A PCR block (Peltier Thermal Cycler, MJ Research) was used for the transcription reaction at 37°C for 60 min. Gene transcript levels were measured by real-time PCR on a Roche Lightcycler 480 II, in 10 μl total volume made up of 4 μl cDNA, 5 μl SYBR Green (Roche), 0.3 μl of each primer (10 μM), and 0.4 μl DEPC treated water (Ambion) using standard conditions for 60 cycles. Target gene expression levels were normalised against the housekeeping gene GAPDH.

Gene	Forward Primer	Reverse Primer	Amplicon Length (nt)
Arg-1	CAGAAGAATGGA AGAGTCAG	CAGATATGCAGGG AGTCACC	249
GAPDH	ATGACATCAAGA AGGTGGTG	CATACCAGGAAAT GAGCTTG	112
IL-25	TGGAGCTCTGCAT CTGTGTC	CGATTCAAGTCCCT GTCCA	114
IL-33	GACACATTGAGC ATCCAAGG	AACAGATTGGTCA TTGTATGTACTCAG	86
MIF	ACAGCATCGGCA AGATCG	GGCCACACAGCAG CTTACT	61
RELMα	TATGAACAGATG GGCCTCCT	GGCAGTTGCAAGT ATCTCCAC	107
RELMβ	GGAAGCTCTCAG TCGTCAAGA	GCACATCCAGTGA CAACCAT	105

Gels, blots and immunoprecipitations

For 2-dimensional separation of HES or HEX, 5-10 µg was mixed with 125 µl rehydration buffer with DTE and IPG buffer added (50 mg DTE (Sigma) and 8 µl IPG buffer pH 3-10 (GE Healthcare, in 1 ml rehydration buffer). Once introduced to a 7 cm ceramic coffin, a 7 cm Immobiline DryStrip pH 3-10 (GE Healthcare) was added and strip oil layered on top, before running on an IPGphor machine 19 hours (20°C 14 hrs, 500V 30 mins, 1000V 30 mins, 8000V 4 hrs). Strips were first equilibrated in 20 mg DTE (Sigma) in 2 ml equilibration buffer, for 15 mins on a rocking platform, followed by 80 mg iodocetamide (Sigma) in the same way. Strips were then run on an IPG well NuPAGE 4-12% BisTris Zoom gel (Life Technologies) for 2 hrs at 100V, with 2 µl SeeBluePlus Standard ladder (Invitrogen), in 1x MES buffer (Life Technologies). 1 ml of 1x MES buffer (Life Technologies) with 0.5% agarose and bromophenol blue (trace) was added on top of the strip before running.

For 1-dimensional gels of fractions, 5 µl of each neat fraction was run down a well of a 15-well NuPAGE 4-12% BisTris gel (Life Technologies), in 1x MES buffer, at 100V for 1-2 hrs.

Silver staining was undertaken by fixing gels (40% ethanol, 10% acetic acid, 50% H_2O) for 30 mins, sensitising (30% ethanol, 0.01M sodium thiosulphate and 0.8M sodium acetate in H_2O) for 30 mins, washing x3 in ultra pure water, staining (15mM silver nitrate in H_2O with 0.02% formaldehyde) for 20 mins, washing x3 in ultra pure water, developing (0.25M sodium carbonate in H_2O with 0.05% formaldehyde) for ~5 mins until proteins are visible, and terminated (20mM EDTA in H_2O) for 5 mins.

Proteins on gels were transferred to 0.45 µm nitrocellulose membranes (BioRad) using 1x NuPAGE transfer buffer (Life Technologies) in a XCell II Blot Module (as per manufacturer's instructions), for 1 hr at 30V. Blots were then washed in 1x TBSt and blocked with 5% milk powder in TBSt for 1 hr at room temperature. Serum was added at 1/1000 dilution in 5% milk solution overnight at 4°C, before polyclonal rabbit α-mouse Igs conjugated to HRP (DAKO) at 1/2000, or HRP-conjugated αIgM (1/1000), or αIgG1 (1/2000) (both Southern Biotech), in 5% milk solution added for 1 hour at room temperature. After washing in TBSt, ChemiGlow West chemiluminescence substrate (ProteinSimple) was added for 20 mins and blots read in a light cabinet (Alpha Inotech). Picture of ladders were taken with white light and overlaid onto the chemiluminescence blot pictures.

For immunoprecipitation of HES with polysera from 4 strains of mice, HES was labeled with biotin (~40 µg biotin reagent/100 µg HES) using EZ-link Sulfo-NHS Biotinylation kit (Pierce) for 2 h on ice and then dialyzed overnight into PBS. Biotinylated HES was then precleared with protein G-agarose beads (16-266; Millipore) in the presence of MOPC 31C IgG1 isotype control for 30 min at room temperature. Unbound HES (2 mg) was then incubated with 5 ml SJL, BALB/c, C57BL/6 or CBA polyclonal serum from day 28 primary infection with *H. polygyrus* in non-denaturing immunoprecipitation (IP) buffer (20 mM Tris [pH 8], 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 1% Triton X-100) for 2 h, and then with protein G-agarose beads overnight at 4°C with rotation. Beads were then washed for 5 min in IP buffer, and bound proteins were eluted with 0.1 M glycine (pH 2.7).

Eluted proteins were buffer exchanged into PBS (with MicroBio-Spin 6 chromatography columns; Bio-Rad), run on one-dimensional and two-dimensional gels and Western blotted as described above, and then probed with 1/2000 streptavidin-horseradish peroxidase (Sigma-Aldrich) before developing to allow visualization of biotinylated proteins. Immunoprecipitation protocol was designed and carried out by Dr James Hewitson.

Monoclonal antibody production

Spleen and MLN cells from d28 *H. polygyrus*-infected C57BL/6 mice, red blood cells lysed as described previously, and mixed in a 1:1 ratio (~$30x10^6$ of each) with SP2 cells, which had been previously grown up from frozen stocks in cRPMI. Cells were spun at 15,000 rpm for 5 mins, and the pellet resuspended. To fuse cells, 1 ml polyethylene glycol was gradually added drop-wise whilst rotating the tube, followed by 1 ml cRPMI in the same way, and the tube made up to 20 ml with cRPMI. After centrifugation at 15,000 rpm for 5 mins, cells were resuspended in 20 ml hybridoma media, transferred to a T75 flask, media added to 200 ml and cells plated in 96-well flat-bottomed plates at 150 µl/well. 100 µl extra hybridoma media was added after 5 days of culture, and wells screened for HES reactivity at day 10 by ELISA - plates coated with 1 µg/ml HES and detected with pan-Ig HRP-conjugated detection antibody (DAKO) at 1:1000. Several positive wells from each plate were transferred to fresh plates and limiting dilutions were done across and down the plate in hybridoma media. Cells were incubated for 10 days and the process repeated twice more to gain monoclonal populations of HES-reactive antibodies. Cells were grown for 10 days in T75 flasks in hybridoma media containing HT supplement in place of HAT, and then adapted to cRPMI for antibody production in Vectra Cell Bioreactors (Bio-Vectra).

Antibody isotypes were determined by ELISA as described above, and purified using an AKTAPrime system and HiTrap protein G HP or HiTrap IgM purification columns (GE Healthcare), as per the manufacturer's instructions.

HES fractionation

500 µg HES was directly injected onto an AKTAExplorer system and separated by size using a gel filtration Superdex 200 10/30 GL 24 ml column or charge using an anion exchange MonoQ 5/50 GL 1 ml column (all GE Healthcare). For separation by size, all fractionation was done in PBS, and the coloumn was previously calibrated with a Gel Filtration HMW Calibration Kit (GE Healthcare) as per the manufacturer's instructions. For anion exchange, HES was first dialysed into start buffer (20 mM Tris-HCl, pH8) with peaks fractionated over a 2-step gradient – 0 - 0.4 M NaCl over 40 ml and 0.4 – 1 M NaCl over 5 ml, by adding elution buffer (20 mM Tris-HCl + 1M NaCl, pH8). All fractionations were eluted in 1 ml fractions into 96-well large-volume plates, using an automated fraction collector. AKTA methods, runs and analysis were designed and controlled using Unicorn software (GE Healthcare).

For analysis of VAL protein content of HES fractions, ELISA plates were coated overnight with 50 µl carbonate buffer containing 0.5 µl of a single fraction per well. Following blocking with 2% BSA in carbonate buffer as described above, plates were probed with α-VAL mAbs or polylonal rat antibodies raised to VAL-1, 2, 3 or 4 (VAL-1 = 4-M15, VAL-2 = 4-S4, VAL-3 = rat pAb, VAL-4 = 2-11), at 5 µg/ml in 50 µl blocking solution for 2 hours at 37°C. Secondary IgG1 conjugated to HRP was added at 1/400 for 1 hour and deteced with ABTS substrate as above.

TGF-β assay

MFB-F11 cells were grown from frozen stocks in 30 ml D10 medium supplemented with 15 µg/ml Hygromycin B (Invitrogen), for 3 days. Confluent cells were detached from the flask with trypsin, and resuspended in D2.5 medium. $4x10^4$ cells were seeded in 100 µl, in 96-well round-bottomed plates. 50 µl of 1:1 HES fraction and PBS, 10 µg/ml HES, serial diluions of recombinant VAL proteins 1-4 (previously made by Yvonne Harcus), or recombinant human TGF-β1 (R&D) in doubling dilutions, starting at 4 ng/ml for use as a standard curve, were added and incubated for 24 hours at 37°C. 10 µl of culture medium was removed onto an

ELISA plate and alkaline phosphatase activity detected using a SEAP Reporter Assay Kit (Invivogen) as per the manufacturers instructions. Plates were read on at 405 nm on an ELISA reader.

Mass spectrometry

~466,000 Roche 454 sequence reads from cDNA of *H. polygyrus* adult and larval stages, and eggs, were assembled into ~20,000 gene products (Harcus *et al*, unpublished). This database, and the NCBI BLAST search tool, were used to match peptides obtained by mass spectrometry (MS) of whole HES, individual HES spots picked from 2-D gels, and TGF-β-positive HES fractions, prepared as described above. MS was undertaken by Adam Dowle at the Technology Facility, Department of Biology, University of York, as described previously (Hewitson *et al* 2008; Hewitson *et al* 2011a; Hewitson *et al* 2011b). Briefly, MALDI mass spectra were obtained and the strongest 10 peaks selected for tandem mass spectrometry fragmentation (MS/MS). MS/MS data were searched against the *H. polygyrus* database using the Mascot program. Peptide matches with expect values <0.05 at a Mowse significance threshold of $p < 0.05$ were considered significant.

Software and statistics

All statistical analyses were performed using Prism 5 (Graphpad Software Inc.). For comparisons of two groups Student's t test was used. When three or more groups were analysed then a one-way ANOVA was used with a Tukey's multiple comparison test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

MacVector 11.1.2 was used to analyse sequences from MS results. SignalP online software was used to predict potential signal sequences, and NCBI BLASTX was used to identify potential protein identities and conserved domains.

Chapter 1

B cell and antibody responses in *H. polygyrus* infection

Introduction

The role of B cells, and antibodies in particular, in protective immunity to helminth infections, remains to be fully established. However, in the case of *H. polygyrus* infection in mice, some conclusive data have been published to show that a protective response to secondary challenge with *H. polygyrus* is dependent on B cells, as μMT and JHD mice (that lack B cells) cannot clear the parasites following challenge after an initial primary infection and anti-helminthic treatment regime (McCoy *et al* 2008; Wojciechowski *et al* 2009; Liu *et al* 2010b). The cellular composition of type-2 intestinal granulomas is comparable in B cell-deficient mice and wild-type mice (Liu *et al* 2010b), suggesting that B cells and antibodies do not play a major role in the formation of granulomas. Moreover, although B and T cell interactions are an important element in an immune response, more than one study found that the defective response to *H. polygyrus* in B cell deficient mice was not due to an impairment of Th2 activation, development or differentiation, and that a pronounced local Th2 response in the intestinal granulomas, and in $CD4^+$ T cells in the MLN, occurred with or without B cells, in both primary and secondary infection (McCoy *et al* 2008; Liu *et al* 2010b). This indicates that B cells are having a role apart from promoting a Th2 response that is vital to worm expulsion in secondary *H. polygyrus* infection. However, another study found that B cells are necessary for an effective Th2 response in both primary and memory responses (Wojciechowski *et al* 2009). These studies use different B cell deficient settings, genetic backgrounds and methodology to assess Th2 cytokine production, which may explain this difference in findings.

Although B cell deficient mice do not make an antibody response, more finely-targeted transgenic mice can be used to elucidate the role of antibody in infection. μs mice have secretory IgM deficient B cells and combining this genotype with AID (activation-induced deaminase) deficiency renders mice unable to undergo affinity maturation or secrete antibody of any isotype. The double-deficient mice were not protected from secondary challenge with *H. polygyrus*, whereas μs mice, that could make parasite-specific class-switched IgG1 and IgE, were able to clear the secondary infection (Wojciechowski *et al* 2009). This finding was corroborated in another study showing that mice that could make class-switched antibody, but that was specific to an irrelevant viral protein, could not control a secondary *H. polygyrus* infection (McCoy *et al* 2008). Using mice deficient in individual antibody isotypes, it was also found that IgE had no role in protection against *H. polygyrus*, and IgA had a minor role, with IgG as the major isotype leading to protection (McCoy *et al* 2008). IgG1 is the most abundant antibody subclass in serum from *H. polygyrus*-infected mice, with levels increasing around 10-fold upon primary infection (Williams and Behnke 1983; Pritchard *et al* 1984) and increasing further still throughout repeated trickle infections – a phenomenon termed hypergammaglobulineamia (Chapman *et al* 1979; Pritchard *et al* 1983; Williams and Behnke 1983). However, protective antibody specificities are only found in serum from mice having been infected with *H. polygyrus* several times, as transfer of naïve or primary serum into C57BL/6 mice does not protect, whereas secondary serum transfer reduces luminal worm numbers by at least two-thirds (Dobson 1982; Williams and Behnke 1983; Pritchard *et al* 1984; McCoy *et al* 2008). This presumably reflects a higher ratio of parasite-specific to nonspecific antibodies in serum from repeated infections. Indeed, the protective effects of serum transfer are seen to be most effective from fractions of serum containing highest parasite-specific IgG1 levels (Pritchard *et al* 1983).

The exact role of antibodies in clearance of an *H.polygyrus* infection is, as yet, unclear. They may act to neutralize antigens in parasite secretions, and prevent them from modulating the immune response. It has been shown that antibodies form immune complexes with HES antigens, clearing them from circulation – higher

levels of HES are found in μMT serum compared with wild-type serum (Herbst *et al* 2012). In this way, antibodies would be working independently of the cellular compartment. However, they may also be acting in concert with immune cells to kill tissue-dwelling stages of the parasite. The granulomatous inflammation around L4 larvae in the submucosa is rich in IgG1 and IgE antibodies and numbers of L4 stage larvae are equal in both primary and secondary infections (McCoy *et al* 2008). It is not until adult parasites have emerged into the lumen, that a protective effect is seen in secondary infection, in C57BL/6 mice (McCoy *et al* 2008), suggesting that mechanisms are acting against the tissue dwelling larval stages rather than adults. The migration of hookworm larvae has been shown to be stalled in mice vaccinated with immune serum (Ghosh and Hotez 1999), leading to the speculation that antibodies may be binding to, and preventing the function of, chemosensory structures or molecules involved in larval migration.

A summary of *H. polygyrus* infection experiments in B cell- and antibody-deficient mouse models is presented in Table 1:

Table 1. *H. polygyrus* in B cell- and antibody-deficient mice

			PRIMARY		SECONDARY			
Model	**Mouse**	**Wild-type**	**Worm count**	**Egg count**	**Worm count**	**Egg count**	**Other observations**	**Reference**
B cell and Ab deficient	**µMT**	C57BL/6	Same	nd	Do not clear	Higher	Transfer of immune serum from C57BL/6 can't rescue in 2° Hp	(Wojciechowski *et al* 2009)
							T cells from 1° Hp, restimulated with Hp-pulsed APCs, make very little Th2 cytokine	(Wojciechowski *et al* 2009)
							µMT can make IgE in absence of IgM and IgD after Hp inf - found bound to basophils	(Perona-Wright *et al* 2008)
	JHD	BALB/c	Same	nd	Do not clear	Higher		(Wojciechowski *et al* 2009)
	JHD	BALB/c	Same	Same	Do not clear	Lower	Eggs may be controlled by B cell-independent factors, in 2° Hp	(Liu *et al* 2010)
							T cell activation not affected by lack of B cells in 2° Hp, and no difference in T reg numbers	(Liu *et al* 2010)
							No defect in Th2 cytokines from MLN T cells or from cells in the granuloma	(Liu *et al* 2010)
	JH$^{-/-}$	C57BL/6	Same	Higher	Do not clear	nd	Comparable T cell responses in 2° Hp	(McCoy *et al* 2008)
							Polyclonal Abs produced in 1° Hp (and present in naïve) act to limit fecundity not worms	(McCoy *et al* 2008)
Abs specific to irrelevant Ag	**MD4µMT (HEL)**	C57BL/6	nd	nd	Do not clear	nd	Diminished Th2 response from T cells	(Wojciechowski *et al* 2009)
	TgH(VI10)xYEN (VSV)	C57BL/6	nd	nd	Do not clear	nd		(McCoy *et al* 2008)
No class switch or somatic hypermutation	**AID$^{-/-}$**	C57BL/6	nd	nd	Do not clear	nd		(McCoy *et al* 2008)
							Basophils but not eosinophils are deficient in AID$^{-/-}$ after 1° and 2° Hp	(Herbst *et al* 2012)
							AID$^{-/-}$ can make HES-specific IgM	(Herbst *et al* 2012)
IgA deficient	**IgA$^{-/-}$**	C57BL/6	nd	nd	Mostly cleared	nd		(McCoy *et al* 2008)
IgE deficient	**IgE$^{-/-}$**	BALB/c	nd	nd	Clear worms	nd		(McCoy *et al* 2008)
Secretory IgM deficient	**µs**	C57BL/6	nd	nd	Clear worms	nd	Can make Hp-specific IgG1/E	(Wojciechowski *et al* 2009)
No affinity maturation or secretion of any Ab isotype	**µs-Aicda$^{-/-}$**	C57BL/6	nd	nd	Do not clear	nd	No Hp-specific Ab	(Wojciechowski *et al* 2009)
µMT + 4getµMT or 4get BM	**µMT+BM**	C57BL/6	nd	nd	nd	nd	4getµMT have fewer IL-4 producing CD4^{+} T cells after 1° and 2° Hp	(Wojciechowski *et al* 2009)
B cells are unable to secrete Ab of any isotype.	**B-µs–Aicda$^{-/-}$ chimeras**	C57BL/6	nd	nd	nd	nd	IL-4Rα^{+} memory T cells still develop after 2° Hp - independent of Ab but needs B cells	(Wojciechowski *et al* 2009)
Various mixed BM chimeras		C57BL/6					IL-4Rα on B cells is needed for protection, but they do not need to be able to produce IL4	(Wojciechowski *et al* 2009)
							B cell derived IL-2 and TNF-α are necessary for establishment of protective immunity	(Wojciechowski *et al* 2009)

N.B. Hp = *H. polygyrus*, 1° = primary, 2° = secondary

Early studies using HES demonstrated antibody recognition of specific dominant immunogenic antigens (Adams *et al* 1987; Adams *et al* 1988; Monroy *et al* 1989a). Indeed, antibodies that recognize components of HES are protective in *in vivo* infection, stunting larval growth (Ey 1988) and preventing adult *H. polygyrus* establishment (Pritchard *et al* 1983). It has been long known that the humoral response is dominated by parasite-specific IgG1 and that serum fractions with higher parasite-specific IgG1 activity afford greater protection when transferred to an infected animal (Pritchard *et al* 1983). Whole serum itself is protective, and transfer from a wild-type donor to a B cell deficient mouse can significantly reduce the number of adult worms left in the intestine after a challenge infection (Liu *et al* 2010b).

More recently, the composition of HES has been systematically analysed and shown to be a complex molecular mixture, with 374 proteins identified by LC-MS/MS (Hewitson *et al* 2011b). Predominantly expressed protein families include proteases, acetylcholinesterases, apyrases and lysozymes (Hewitson *et al* 2011b; Moreno *et al* 2011). However, the most abundant family is VALs, of which 25 members were found in HES (Hewitson *et al* 2011b). They are highly divergent, and found in a range of other parasitic helminth ES preparations including those of *Haemonchus* (Yatsuda *et al* 2003), *Ancylostoma* (named *Ancylostoma*-secreted protein, or ASP) (Hawdon *et al* 1996), *Brugia* (Hewitson *et al* 2008) and *Teladorsagia* (Craig *et al* 2006). However, no known function for VALs has been attributed.

As described, most studies on the role of antibody in *H. polygyrus* have focused on secondary infections and on the parasitological outcome. Here, the impact of a number of B cell and antibody deficiencies on the outcome of primary infection were investigated and the immunodominant antigens contained in HES were identified and used to further characterize the antibody responses to infection with *H. polygyrus.*

Results

1.1 µMT mice are fully susceptible to *H. polygyrus* infection and fail to form granulomas around invading larvae

Mice that lack B cells and antibodies completely, on both BALB/c and C57BL/6 genetic backgrounds, have been shown to be unable to expel secondary challenge infections with *H. polygyrus* (McCoy *et al* 2008; Wojciechowski *et al* 2009; Liu *et al* 2010b). µMT mice have a disrupted gene encoding the membrane form of the µ-chain constant region, thus in homozygous mice, B cells can not develop to perform effector functions and produce antibodies (Kitamura *et al* 1991). In primary infection, at day 14 post-infection with *H. polygyrus,* there was no difference in the number of parasites in the lumen of the small intestine between µMT mice and C57BL/6 wild-type mice (Fig 1.1 A). By day 28 there was a trend for greater numbers of worms in the B cell-deficient mice (Fig 1.1 B, data collected by Dr Katie Smith). Furthermore, a striking finding was that µMT mice could not form intestinal granulomas, and at day 14 post-infection had nearly none compared to the numbers present in C57BL/6 mice (Fig 1.1 C).

In these initial experiments, egg production was not measured, although this may be a more sensitive measure of immunological differences. In other studies with *H. polygyrus* in B cell-deficient mice this parameter has yielded interesting results. In primary infection in JHD mice, worm and egg counts are only slightly higher than in wild-type BALB/c at day 14, which is before most parasites are cleared in this genetic background (Liu *et al* 2010b). The same study also showed that immunity to challenge infection failed to expel adult worms in JHD mice, although egg counts were greatly decreased, prompting the authors to suggest that anti-fecundity effects are B cell independent (Liu *et al* 2010b).

1.2 MD4 mice are unable to produce parasite-specific antibodies, and are fully susceptible to *H. polygyrus* infection

MD4 mice are BCR-transgenic and make a monoclonal repertoire of antibodies that only recognize an irrelevant foreign protein. A transgenic construct containing rearranged heavy and light chain genes encoding high-affinity anti-hen egg lysozyme antibodies was made, and in the presence of this rearranged genomic insert, endogenous rearrangement is suppressed and the mice can only express anti-HEL immunoglobulins (Goodnow *et al* 1988). Previous studies, including some experiments utilizing MD4 mice, have shown that protective immunity following secondary infection with *H. polygyrus* is dependent on parasite-specific antibodies (McCoy *et al* 2008; Wojciechowski *et al* 2009). While worm burdens (Fig 1.2 A) and egg counts (Fig 1.2 B) in MD4 mice, 28 days following primary *H. polygyrus* infection, were higher than in BALB/c wild-type mice, the differences did not reach statistical significance. However, there was clearly no difference in the number of granulomas found on the wall of the small intestine (Fig 1.2 C), indicating that parasite-specific antibodies are not involved in the formation of these type-2 inflammatory foci found uniquely in *H. polygyrus* infection (Patel *et al* 2009).

To measure parasite-specific serum antibodies, an ELISA was used with the adult excretory-secretory (HES) antigens as detailed below. This assay, detecting all antibodies that bound to HES, confirmed that only BALB/c mice produced HES-specific antibody following 28 days of *H. polygyrus* infection (Fig 1.2 D). Only antibodies of an IgD or IgM isotype can be made by MD4 mice, due to insertion of the genetic construct replacing the γ1 constant region on the heavy chain gene (Goodnow *et al* 1988). Total serum IgM was therefore also measured after *H. polygyrus* infection, and levels were found to be similar between BALB/c and MD4 mice (Fig 1.2 E). This reinforces the studies that show parasite-specific IgG1 is only protective in secondary, and subsequent infections. Although parasite numbers were slightly higher in MD4 mice, which cannot make *H. polygyrus*-specific IgG1, in primary infection, the defect in immunity was not as profound as in secondary infection (Wojciechowski *et al* 2009).

1.3 CD40$^{-/-}$ and CD154$^{-/-}$ show no difference in worm burden after primary infection to wild-type C57BL/6

Finally, the effect of CD40 and CD154 (CD40L) deficiency on the response of mice to a primary infection of *H. polygyrus* was investigated. The interaction of these co-stimulatory molecules, along with numerous other signals, induces B cell clonal expansion, antibody synthesis and isotype switching (Bishop and Hostager 2003; Subauste 2009). Human defects in either molecule result in serious immunodeficiencies, in which antibodies do not class-switch and therefore patients are left susceptible to opportunistic infections (Allen *et al* 1993; Durandy and Kracker 2012). Disruption of CD40 and CD40L interactions with antibodies, during primary *H. polygyrus* infection in mice, resulted in lower total IgG1 in the serum and reduced blood eosinophilia (Lu *et al* 1996). However, treatment did not inhibit increases in transcripts of Th2 cytokines in the MLN or PP following infection, or IL-4 production from cultured MLN lymphocytes *in vitro* (Lu *et al* 1996). Conversely, in *Schistosoma mansoni*-infected mice, CD40-CD154 interactions have been found crucial to the instigation of a protective Th2 response and the resulting worm killing (Subauste 2009).

By day 28 post-infection with primary *H. polygyrus*, neither CD40$^{-/-}$ nor CD154$^{-/-}$ showed a difference in susceptibility to the parasite, compared to C57BL/6 wild-type mice (Fig 1.3 A). This was also reflected in the faecal egg count from this time point (Fig 1.3 B). Both CD40$^{-/-}$ and CD154$^{-/-}$ genotypes were confirmed to have defects in class-switching, as the levels of HES-specific IgG1 in the serum of these mice were non-existent at both days 14 and 28 after infection, compared to C57BL/6 which display 5-50-fold increases in IgG1 titre over time (Fig 1.3 C). They could however make HES-specific IgM, although to only 10-20% of the levels of C57BL/6 mice (Fig 1.3 D). At day 14 all three groups of mice made detectable levels of HES-specific IgM, with a decrease by d28 in C57BL/6, presumably reflecting the class switch to IgG1 and other isotypes of antibody (Fig 1.3 D). In contrast to a published study using antibodies to block signalling through CD40 and CD154 in which type-2 cytokine responses to *H. polygyrus* are undiminished (Lu *et al* 1996), it was found here that a genetic deficiency in these molecules compromises the Th2 response

following infection. Thus, levels of IL-4 and IL-13 from MLN cells polyclonally stimulated with α-CD3 were significantly lower in $CD40^{-/-}$ and $CD154^{-/-}$ mice than in the wild-type C57BL/6 controls (Fig 1.3 E).

1.4 Primary antibody responses are predominantly directed at secreted, rather than somatic, parasite antigens

To investigate the specificity of antibody responses in *H. polygyrus* infection, 2 antigenic preparations were characterized by staining 2-dimensional SDS gels with silver nitrate, showing the distinct profile of proteins found in HES compared to soluble worm extract (HEX) (Fig 1.4 A, B). This was confirmed by proteomic analysis by LC-MS/MS, which showed of the 374 proteins identified in HES, only 28% could be found in HEX, demonstrating that HES represents a selective subset of the complete proteome of the adult worm (Hewitson *et al* 2011b).

Polyclonal antibodies in serum were first investigated, using pooled sera from C57BL/6 mice, infected for 28 days with *H. polygyrus*. These antibodies bound to a very restricted set of antigens in HES, shown on a 2D Western blot (Fig 1.4 C), previously identified as VAL-1, 2 and 5 by proteomics (Hewitson *et al* 2011b). In contrast, very few HEX antigens were bound by antibodies (Fig 1.4 D, F). For this reason, further studies tested only HES as it contains the most immunodominant antigens which are not well represented in HEX.

Mice with primary infections extending to 100 days did not expand the repertoire of proteins bound by antibodies, but did increase intensity of binding, indicating a higher titre of HES-specific antibody later in the infection (Fig 1.4 E). At this time point C57BL/6 mice (which are classed as slow responders to *H. polygyrus* infection) retain low numbers of adult parasites in the lumen of the small intestine (data not shown).

1.5 Generation of monoclonal antibodies to HES antigens

A panel of monoclonal antibodies (mAbs) was made to analyse the antigen specificity of the antibody response to primary *H. polygyrus* infection further. Splenocytes and MLNCs from d28-infected C57BL/6 mice were fused to Sp2 hybridoma cells, and screened for HES reactivity by ELISA (see Fig 1.5 A). Positive wells were picked at each screen and serially diluted by limiting dilutions to select monoclonal populations. Even at day 28 post-infection, a large proportion of antibodies to HES were of an IgM isotype, especially among monoclonals derived from splenic B cells (Fig 1.5 B), while MLN-derived mAbs were all IgG1 class-switched antibodies (Fig 1.5 C). A summary of all mAbs that were successfully expanded and analysed is presented in Table 1.2.

1.6 The immunodominant antigens in HES are VAL glycoproteins

The majority of IgM HES mAbs bound to proteins previously identified as VAL-1, 2 and 5 (Hewitson *et al* 2011b), in Western blot profiles very similar to that of polyclonal primary serum (Fig 1.6 A-C). Although this indicates binding to a common immunodominant epitope on VAL-1, 2 and 5, the sequence homology of these three proteins is low (approx. 15% identity) (Hewitson *et al* 2011a). Further biochemical analysis (carried out by Dr James Hewitson and published in (Hewitson *et al* 2011a)) showed that the immunodominant common epitope recognized by the majority of IgM mAbs was an O-linked glycan.

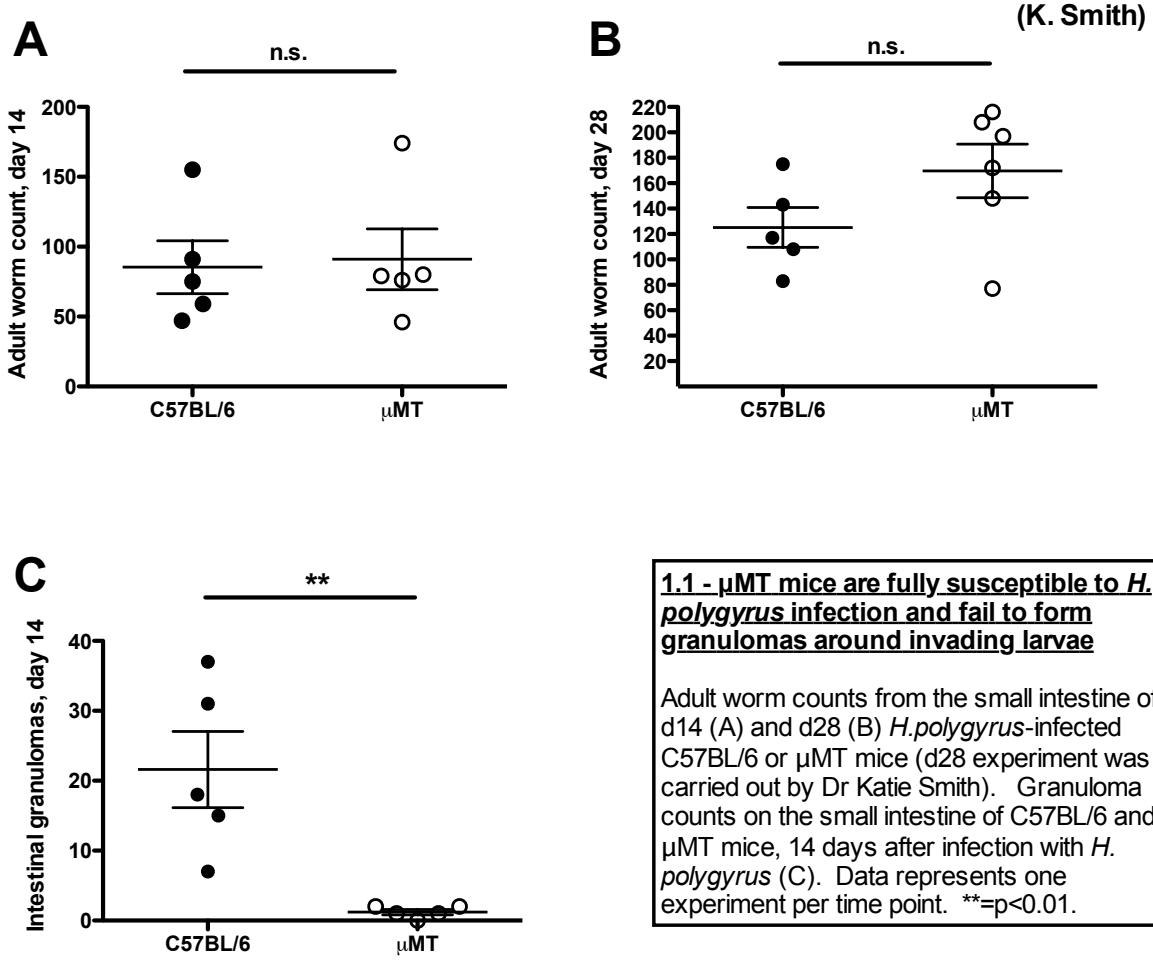
Remarkably, most IgG1 mAbs failed to bind to HES on Western blots, despite their strong reactivity to HES by ELISA. This was an indication that they recognized heat-labile conformational epitopes that are sensitive to denaturation during the Western blot protocol. Therefore antibodies were used to probe biotin-labelled non-denatured HES by immunoprecipitation with protein G beads, prior to 2-D electrophoresis and staining with streptavidin-peroxidase. By this technique, the majority of IgG1 mAbs were shown to bind to VAL-1 (Fig 1.6 D, E), while two additional IgG1 mAbs were shown to bind to proteins at the positions of VAL-2 and VAL-4 (Fig 1.6 F, G).

Table 1.2 - mAbs generated from primary infection with *H. polygyrus* in C57BL/6 mice

Antigen Specificity	*Clone ID*	*Source*	*Isotype*	*Heat Stable/Labile Epitope*
VAL-1, 2 & 5 common epitope	13.1*	Spleen	IgM	Stable
	2-2			Stable
	2-12			Stable
	2-13*			Stable
	2-62			Stable
	3-8			Stable
	3-11			Stable
	3-28			Stable
	3-29			Stable
	3-40			Stable
	3-42			Stable
	3-55*			Stable
VAL-1	2-6*	Spleen	IgG1	Labile
	3-6			Labile
	3-10			Labile
	3-38			Labile
	3-39			Labile
	4-M4	MLN	IgG1	Labile
	4-M15*			Labile
	4-M20			Labile
	4-M23			Labile
	4-M25			Labile
VAL-2	4-S4*	Spleen	IgG1	Labile
VAL-4	2-11*	Spleen	IgG1	Labile

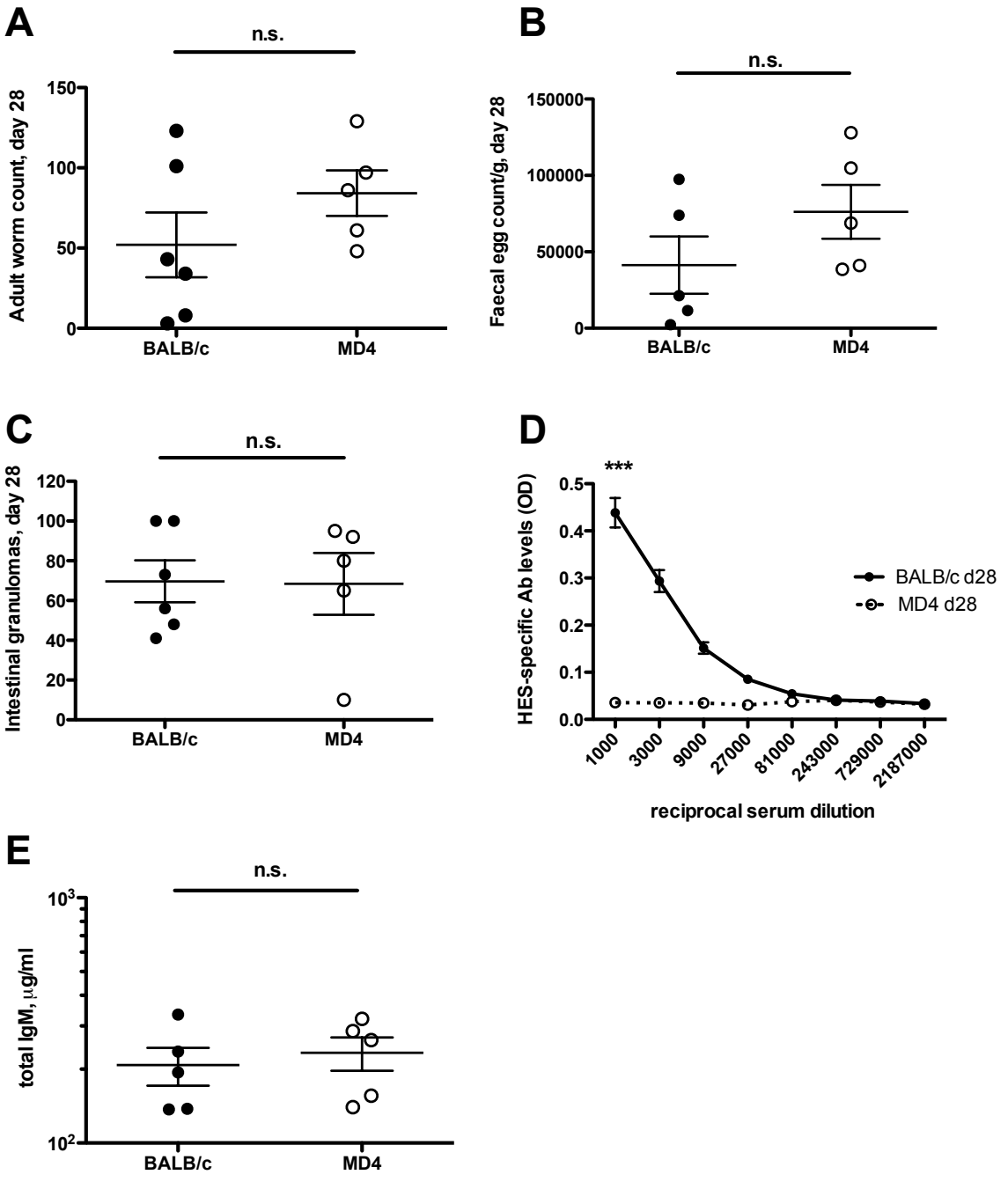
* = Western blot is represented in Fig 1.6.

(K. Smith)



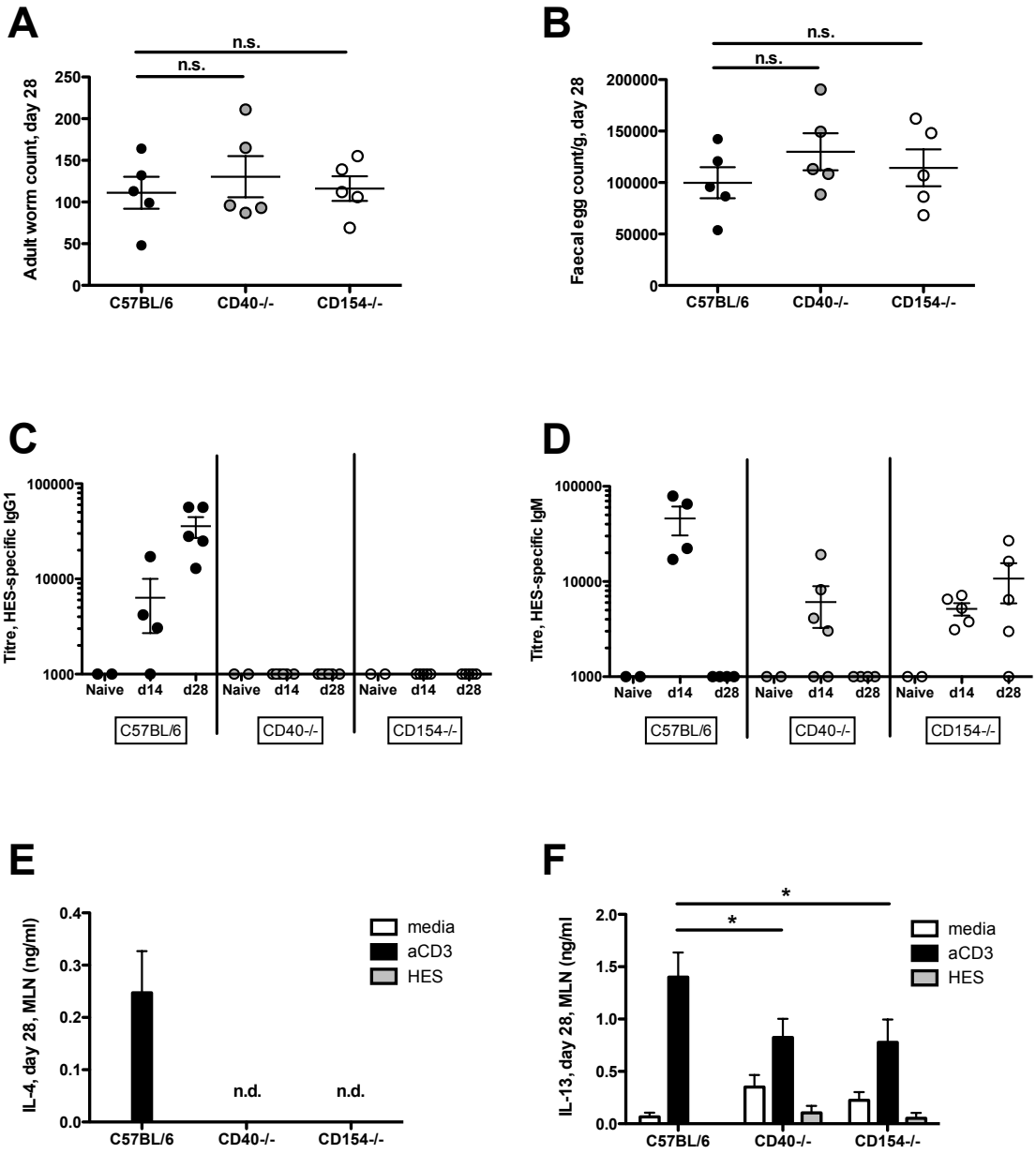
1.1 - µMT mice are fully susceptible to *H. polygyrus* infection and fail to form granulomas around invading larvae

Adult worm counts from the small intestine of d14 (A) and d28 (B) *H.polygyrus*-infected C57BL/6 or µMT mice (d28 experiment was carried out by Dr Katie Smith). Granuloma counts on the small intestine of C57BL/6 and µMT mice, 14 days after infection with *H. polygyrus* (C). Data represents one experiment per time point. **=p<0.01.



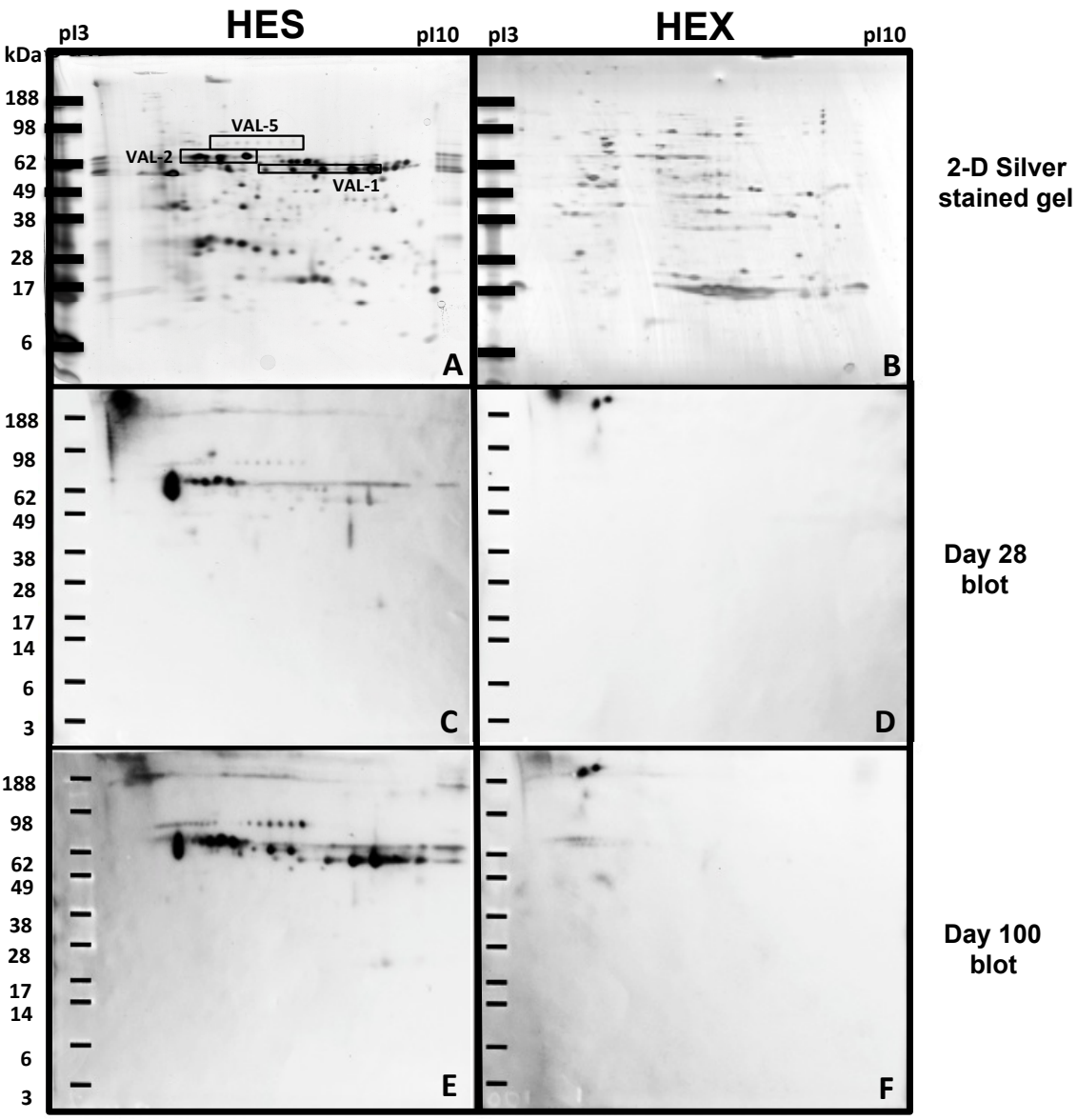
1.2 - MD4 mice are unable to produce parasite-specific antibodies, and are fully susceptible to *H. polygyrus* infection

Adult worm counts (A) and faecal egg counts (B) from the small intestines of d28 *H.polygyrus*-infected BALB/c or MD4 mice. Granuloma counts on the small intestine of BALB/c and MD4 mice, 28 days after infection with *H. polygyrus* (C). Binding of serum antibodies to HES on an ELISA plate measured at OD 280, with serum from d28 infected BALB/c (closed circles) or MD4 (open circles) mice (D). Total amounts (µg/ml) of IgM antibodies in the serum of individual BALB/c and MD4 mice (E&F). Data represent one experiment. *=p<0.05, ***=p<0.001.



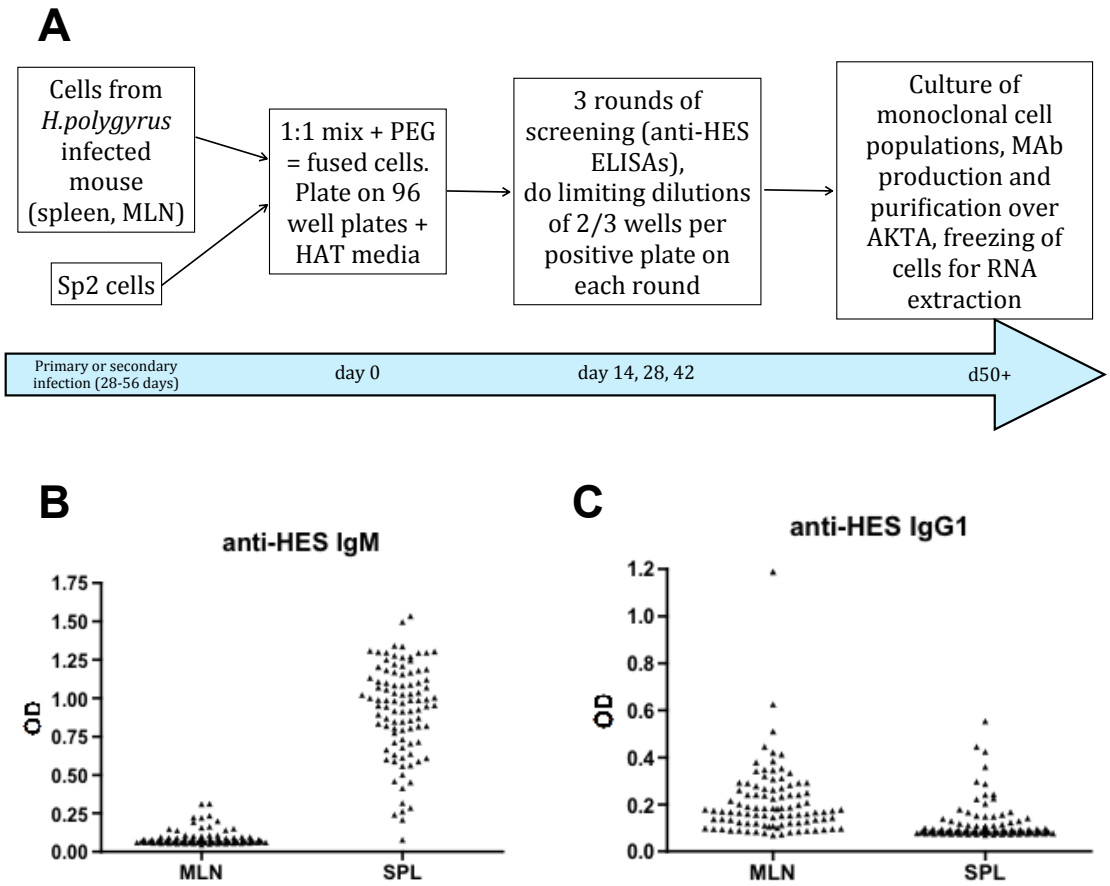
1.3 - CD40$^{-/-}$ and CD154$^{-/-}$ show no difference in worm burden after primary infection to wild-type C57BL/6

Adult worm counts (A) and faecal egg counts (B) from the small intestine of d28 *H.polygyrus*-infected C57BL/6, CD40$^{-/-}$ and CD154$^{-/-}$ mice. Titres of HES-specific IgG1 (C) and IgM (D) in serum from naïve, d14 and d28-infected mice. Levels of IL-4 (E) and IL-13 (F) released from d28-infected mouse MLN cells stimulated with media, HES and α-CD3 for 3 days *in vitro*. Results are representative of 2 independent experiments. *=p<0.05, **=p<0.01.



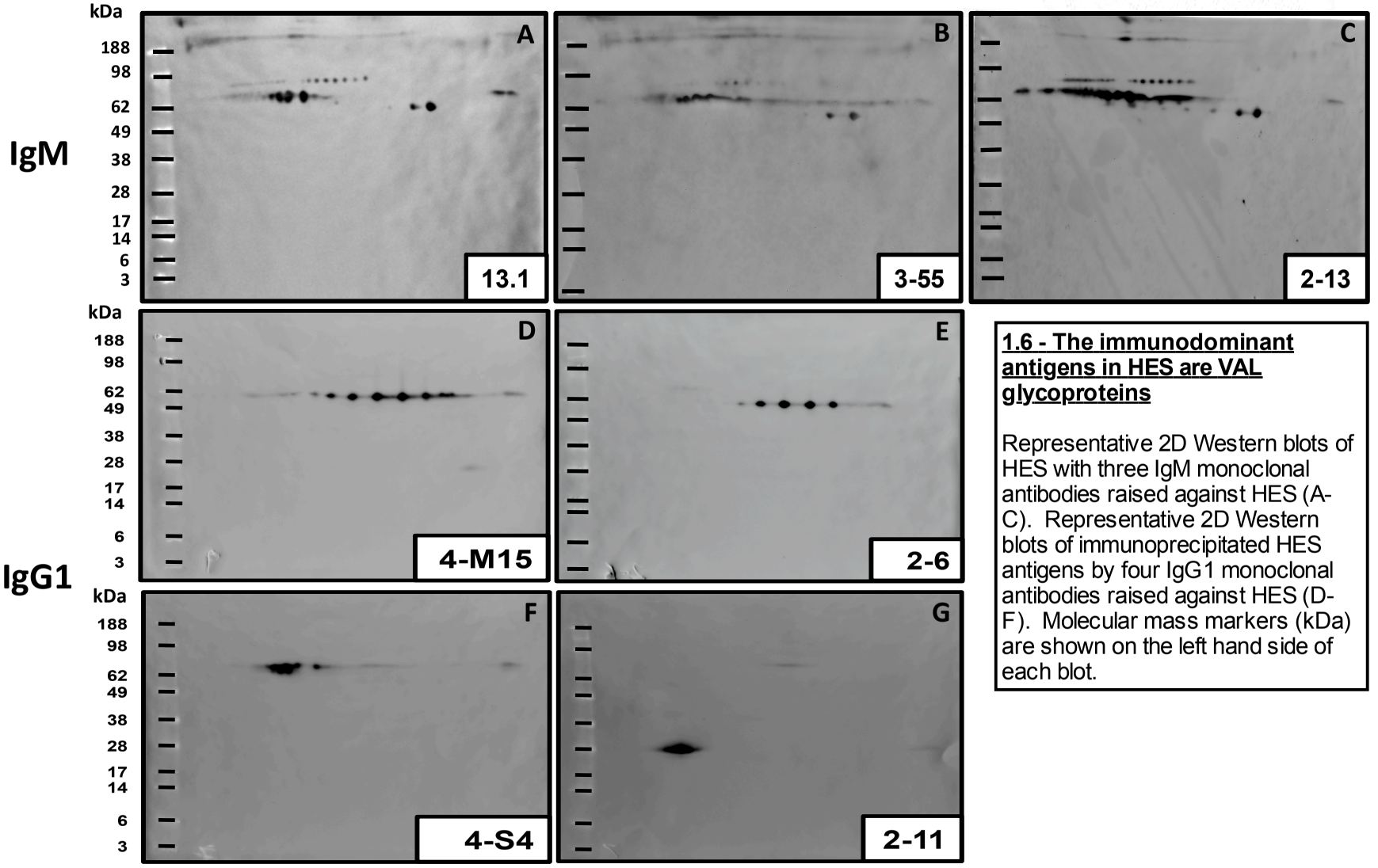
1.4 - Primary antibody responses are predominantly directed at secreted, rather than somatic, parasite antigens

10µg of HES (A) or HEX (B) were run in the first dimension along an isoelectric focusing strip, and then down a conventional SDS-PAGE gel. Subsequent silver staining revealed protein content. Molecular mass markers (kDa) are shown on the left hand side of each gel. The major VAL components are indicated having previously been identified with LC-MS/MS (Hewitson et al. 2011). Western blots of 2D gels of HES and HEX with serum from d28 infected (C&D) or d100 infected (E&F) C57BL/6 mice. Blots were detected using a polyvalent anti-Ig conjugate. Molecular mass markers (kDa) are shown on the left hand side of each blot. Serum is pooled from 5 mice per time point and results are representative of two or more experiments.



1.5 - Generation of monoclonal antibodies to HES antigens

Schematic showing the workflow for generation of monoclonal antibodies to HES components (A). Dot plots showing the frequency of IgM and IgG1 monoclonal antibodies generated to HES components from either spleen or MLN cells from d28 *H. polygyrus*-infected C57BL/6 mice (B&C). Each dot represents a single positive well from a 96-well plate screen.



1.6 - The immunodominant antigens in HES are VAL glycoproteins

Representative 2D Western blots of HES with three IgM monoclonal antibodies raised against HES (A-C). Representative 2D Western blots of immunoprecipitated HES antigens by four IgG1 monoclonal antibodies raised against HES (D-F). Molecular mass markers (kDa) are shown on the left hand side of each blot.

Discussion

B cells are a key cell type in the development and successful mediation of a Th2 response against helminths. As well as the production of antibodies, both parasite-specific and nonspecific, that can act directly against the parasite, and augment cellular immunity themselves, B cells can have antibody-independent effects, such as antigen presentation and cytokine production (Harris and Gause 2011). Although several studies in B cell and antibody deficient mice have highlighted the importance of B cells and antibodies in secondary challenge infection with *H. polygyrus* infection, primary infection has not been studied to the same extent. Use of primary infection gives a picture of how the immune system initially recognizes the presence of the worm, and also prevents the complication of the use of anti-helminthic drugs, which may release internal antigens from the worm that natural infection would not normally expose.

MD4 mice can not make HES-specific antibodies, although this had only a small effect on primary day 28 worm burden and egg production, reinforcing the findings from others (McCoy *et al* 2008) that the parasite-specific antibodies raised in wild-type mice are either of low affinity, target molecules that are not essential to parasite survival, or at a too low a level to have a detrimental effect. B cell-deficient μMT mice, and those which have defects in either CD40 or CD154 (and therefore class-switching and T cell-dependent antibody production), have slight, but not significant, defects in the ability to clear worms and curb egg production in a primary infection, by day 28. This phenotype has been noted in other B cell deficient mice on both a C57BL/6 and BALB/c background (Wojciechowski *et al* 2009; Liu *et al* 2010b). It is unclear why differences in Th2 cytokines and antibodies do not translate to a greater difference in worm burden in $CD40^{-/-}$ and $CD154^{-/-}$ mice. C57BL/6 are known as slow responders in *H. polygyrus* infection and, compared to other strains such as BALB/c, still have high numbers of worms at day 28 in a primary infection. Therefore, an even later time point might reveal differences in ability to expel the worm in the knock-out mice, and the use of gene knock-outs on a BALB/c

background may be more appropriate, as any difference in ability to clear adult parasites will be amplified in these mice.

μMT mice did not form granulomas in response to *H. polygyrus* infection. Although B cells are not a key cell type in granulomas formed around *H. polygyrus* (Patel *et al* 2009), the presence of antibodies bound to the surface of both the larvae and the inflammatory cells forming the granuloma may act to activate cells and complement cascades to induce further cell infiltration and cytokine production (Harris and Gause 2011). In no study with *H. polygyrus* in B cell or antibody deficient mice, have granuloma numbers been quantified. One study did extract RNA from granulomas isolated from the gut wall, and found there to be no difference in the Th2 cytokine transcript in granuloma cells between JHD and BALB/c wild-type mice (Liu *et al* 2010b).

The importance of HES as a key factor at the interface between parasite and host immune system is demonstrated by its ability to trigger strong antibody responses in comparison to HEX. Previous studies have identified the most immunodominant antigens in HES to be in the range of 50-70 kDa (Monroy *et al* 1989d; Pleass and Bianco 1996; Ben-Smith *et al* 1999) – the experiments presented here identify these antigens as members of the VAL family, to which mAbs raised from infected mice also bind. We found subsets of mAbs that recognize either a common carbohydrate epitope on VALs, or the heat-labile polypeptide structure of individual VALs, broadly split by antibody isotype. Why some epitopes are more immunodominant than others may rely on several factors. It is interesting to consider that the majority of IgM mAbs we made recognized a common epitope across several different VAL molecules and were shown to bind the adult worm cuticular surface (Hewitson *et al* 2011a). Therefore these specificities of antibodies may simply represent an evolutionarily derived first-line of defence, before more defined, class switched antibodies can be made to target individual molecules released by the worm.

Antibodies may act in several ways. Although primary infection serum has not been found to be protective in terms of worm number reduction (Pritchard *et al* 1984; McCoy *et al* 2008), the reduction in fecundity that was documented (McCoy *et al* 2008) would act in the wild, to minimise further infection of the host population. Neutralisation of parasite-derived molecules by antibodies may act to limit larval growth and development (Ey 1988), or direct molecular and cellular immunity against the adult worm surface. It has previously been shown that IgG1 antibodies are the most dominant, and protective when transferred (Pritchard *et al* 1983), and we found the majority of class-switched antibodies to be of this isotype. Indeed, evidence for the protective effects of IgG1 antibodies is presented in studies with different strains of mice that respond to *H. polygyrus* to varying degrees – 'fast-responder' strains (SJL and SWR) have a quicker and stronger antibody response, and recognize a greater range of antigens, than 'slow-responders' (CBA and C57BL/10) (Ben-Smith *et al* 1999).

The mAbs raised to HES were tested for their ability to limit parasite numbers and egg production, but none conferred protection (Hewitson *et al* 2011a). This is in contrast to vaccination with whole HES, which results in a complete reduction in worm and egg numbers compared to PBS controls (Hewitson *et al* 2011a). Single mAbs were used in the transfer experiments, and it may be that combinations of specificities are needed for immunity. Another question may be the availability of the antibodies to the worm. IgG1 antibodies have been found in the intestinal lumen following *H. polygyrus* infection (Ben-Smith *et al* 1999), but it is unclear whether IgM antibodies could actually gain access to the worm in the intestine, from the circulation. There is also a possibility that the parasite may make proteins with conserved epitopes that are in fact a decoy, directing antibody responses to harmless antigens, and away from those that may be crucial to the survival of the worm (Maizels *et al* 1993).

The identification of immunodominant molecules in HES could give insight into how *H. polygyrus* can manipulate the host response, and if components of HES could be used therapeutically in the future.

Key Findings

- In accordance with published literature, the ability of mice to expel adult worms by day 28 in a primary *H. polygyrus* infection is unaffected by lack of B cells, specific antibodies or ability to class switch, although using mice on a more resistant background may yield more significant differences between wild-type and deficient mice.
- Surprisingly, μMT mice cannot form intestinal granulomas around tissue-invading larvae, a characteristic feature of *H. polygyrus* infection. However, this does not impede expulsion of the parasite. This is an unexpected, and unreported finding, and could be investigated further.
- The primary antibody response to *H. polygyrus* infection is restricted to a very small subset of the proteins in HES and is primarily IgM and IgG dominated.

Chapter 2

Immune mechanisms involved in immunity to primary infection with *H. polygyrus*

Introduction

Studies in both mouse and human populations strongly indicate that immunity to gastrointestinal nematode parasites requires a strong Th2 responsiveness profile (Turner *et al* 2003; Finkelman *et al* 2004; Jackson *et al* 2004) with the canonical type 2 cytokines IL-4 and IL-13 critical in mobilising a range of innate effector mechanisms that disable and expel gut helminths (Else *et al* 1994; Finkelman *et al* 1997; Hayes *et al* 2004; Anthony *et al* 2007; Maizels *et al* 2012). Interestingly, human populations show a spectrum of responses to helminth infection, varying from effective resistance through hyporesponsiveness and tolerance, to hyperreactivity and pathogenesis (Maizels and Yazdanbakhsh 2003). Allelic variation at key loci controlling type 2 cytokines and their signals (such as IL-13 and STAT6) is an important genetic factor influencing the outcome of helminth infection in humans (Peisong *et al* 2004; Kouriba *et al* 2005), and likewise, different strains of mice can display contrasting patterns of susceptibility or resistance to helminth parasites. Hence, mouse models can provide new insights not only into immunological mechanisms of protection, but also the genetic basis for variation in the efficacy of those mechanisms.

The rat parasite *N. brasiliensis* is rapidly expelled by Th2-dependent mechanisms in all immunocompetent strains of mice (Urban *et al* 1998). A more balanced picture is seen with the cecal-dwelling *Trichuris muris*, in which Th2-dependent immunity is directly antagonised by the degree of Th1 responsiveness (Hayes *et al* 2004). Thus, blocking IFN-γ responses enables a susceptible mouse to clear infection (Else *et al* 1994) while exogenous IL-12 prolongs infection in a genotypically resistant animal (Bancroft *et al* 1997). A further layer of complexity is observed with *H. polygyrus*,

which generates a significant regulatory T cell population that inhibits Th2 immunity (Wilson *et al* 2005; Finney *et al* 2007; Setiawan *et al* 2007; Rausch *et al* 2008; Grainger *et al* 2010). Recent data implicate similar regulatory effects in human intestinal helminth infections (Turner *et al* 2008; Figueiredo *et al* 2010) suggesting that *H. polygyrus* may offer a valuable system to model such interactions.

A further feature of the *H. polygyrus* model of immunity is that inbred strains differ markedly in their ability to expel primary infections (Prowse *et al* 1979; Enriquez *et al* 1988; Wahid *et al* 1989; Wahid and Behnke 1993b; Prowse and Mitchell 2005; Behnke *et al* 2009)(see Table 2.1). Resistant mouse strains such as SJL show faster and stronger antibody and Th2-type responses (Wahid and Behnke 1993b; Lawrence and Pritchard 1994), but as yet few details are available which compare T cell subsets or innate immune components between strains with differing capacity to reject primary infection. In addition, while resistance in previously immunized mice is associated with formation of granulomas around encysted larvae (Anthony *et al* 2006; Patel *et al* 2009), the role of granulomas in primary immunity has not been evaluated.

With the aim of further characterising important aspects of immunity to *H. polygyrus*, the strength of both innate and adaptive immune responses were correlated with ability to expel the worm by four widely used mouse strains, SJL, BALB/c, C57BL/6 and CBA. These responses included T-cell cytokines, T regulatory cells, antibodies, innate lymphoid cells and alternatively-activated macrophages. In addition, the granuloma was investigated more fully with regards to frequency, cellular composition and role in immunity, as this has not been fully investigated in a primary setting.

Table 2.1 – 'Responsiveness' of inbred mouse strains to *H. polygyrus*

'Responsiveness'	Strain	Immune mechanisms investigated
Slow (>20 weeks to expel worms)	CBA C3H SL A/J	Primary response stimulates significantly lower cell numbers in the MLN than other strain (Parker and Inchley 1990; Lawrence and Pritchard 1994), very few mast cells in the gut (Lawrence and Pritchard 1994), low levels of mMCP in serum and intestinal lavage (Behnke *et al* 2003b; Ben-Smith *et al* 2003), and low eosinophilia (Zhong and Dobson 1996). Have no, or very weak, protective response to re-challenge (Behnke *et al* 1983; Behnke and Robinson 1985; Behnke *et al* 2003b).
Intermediate (8-20 weeks)	C57BL/6 C57BL/10 129/J	C57BL/10 mice show less rapid and lower eosinophilia levels in circulation, after both primary *H. polygyrus* infection or injection of parasite antigens, than NIH mice (Wakelin and Donachie 1983).
Fast (6-8 weeks)	DBA/2 BALB/c NIH	NIH mice produced a higher peak of lymphocytosis, neutrophilia and monocytosis in the circulation than C57BL/10 mice after primary infection (Ali *et al* 1985). NIH, BALB/c and DBA/2 mice have more effective protection after multiple infections than C57BL/6, C57BL/10 and CBA (Behnke and Robinson 1985; Enriquez *et al* 1988). NIH and BALB/c mice have more intense IgG1 responses than C57BL/10, with higher titres noted after infection with a larger inoculum of larvae (Wahid and Behnke 1993a). BALB/c have higher numbers of B and T cells in the MLN throughout infection, than CBA mice (Lawrence and Pritchard 1994).
Rapid (4-6 weeks)	SJL SWR	SJL and SWR have quicker and stronger antibody responses than other strains, involving stronger recognition of a larger number of antigens on a Western blot of HES (Ben-Smith *et al* 1999) and adult worm homogenate (Lawrence and Pritchard 1994), and higher titres of parasite specific antibody of different isotypes in serum (Lawrence and Pritchard 1994; Ben-Smith *et al* 1999; Ben-Smith *et al* 2003). Infected SWR MLN cells produced higher levels of IL-3, IL-4 and IL-9 after ConA stimulation than NIH and CBA (Wahid *et al* 1994). Both strains show early peaks of serum TNF, mMCP-1, intestinal mast cells and goblet cells, which precede the expulsion of the worms (Behnke *et al* 2003b; Ben-Smith *et al* 2003).

Results

2.1 Variation in susceptibility to primary infection with *H. polygyrus* manifests first with early differences in fecundity and granuloma formation, and subsequent loss of adult worms

Inbred mouse strains are known to vary significantly in their susceptibility to *H. polygyrus* (Prowse *et al* 1979; Behnke *et al* 2009) and in comparing 4 mouse strains three critical features of differential immunity were noted. First, luminal adult worms were present in all groups at day 14, soon after emergence (Fig. 2.1 A), and only subsequently did genetically determined differences in expulsion become apparent. Thus, by day 28 resistant strains had expelled most of the adult parasites, with BALB/c and SJL mice carrying the lowest numbers while C57BL/6 and CBA retained high numbers (Fig. 2.1 B).

Secondly, at the earlier time point of day 14, parasite egg production was much lower in relatively resistant SJL mice than in the fully susceptible CBA and C57BL/6 strains (Fig. 2.1 C). As the total number of adult worms in the gut lumen was similar in all groups at this time point (Fig. 2.1 A), this phase of immunity represents a reduction in worm fitness as reflected by their fecundity. By day 28, egg production more closely mirrored adult worm loads with CBA and C57BL/6 mice excreting the most, and BALB/c and SJL the fewest (Fig. 2.1 D).

Thirdly, the more resistant strains showed more extensive development of macroscopic granulomas in the intestinal wall (Fig. 2.1 E-G), which although numerous in the more resistant genotypes are sparse in the fully susceptible mice (Fig. 2.1 H). The abundance of granulomas shows, in this day 14 comparison, an inverse relationship with worm fecundity (Fig. 2.1 I) suggesting that they may impair fitness of the parasite while in the intestinal wall.

2.2 The most susceptible strains make a weak Th2 response to *H. polygyrus*, which is counterbalanced by release of IFN-γ by T cells

Cellular immune responses were first assessed at day 7 post-infection using both polyclonal and antigen-specific assays. *In vitro* stimulation of draining MLN cells with *H. polygyrus* ES material (HES) and anti-CD3 antibody for 72 hours elicited high levels of IL-4, IL-13 and IL-10 from SJL and BALB/c, measured by ELISA (Fig. 2.2 A-C). In the same assays, susceptible C57BL/6 and CBA mice mounted weaker Th2 responses, while expressing higher levels of IFN-γ (Fig. 2.2 D). Direct intracellular cytokine staining for IFN-γ also showed significantly higher levels in both $CD4^+$ (Fig 2.2 E) and $CD8^+$ T cells (Fig 2.2 F) from the most susceptible strains. CBA mice in particular made extremely poor T cell IL-4 responses compared to the other 3 strains measured in this way (Fig 2.2 G).

These results concur with previous findings showing that a strong Th2 response is correlative with resistance to *H. polygyrus* (Wahid *et al* 1994). It has been shown in several publications that regulatory T cells induced by *H. polygyrus* are responsible for downregulation of IFN-γ, which can often prevent immune pathology (Bazzone *et al* 2008; Ince *et al* 2009; Tetsutani *et al* 2009). A predisposition of C57BL/6 and CBA mice to overexpress IFN-γ (Fig 2.2 D-F), and produce low levels of regulatory cytokines such as IL-10 (Fig 2.2 C) would fit with findings that susceptibility to *H. polygyrus* correlates with higher levels of Th1 cytokines after removal of regulatory systems (Reynolds and Maizels 2012). CBA mice in particular displayed very poor Th2 responses to HES and to polyclonal stimulation with αCD3, which were significantly lower than in SJL and BALB/c mice (Fig 2.2 A-B), which may be due to the over-exuberant production of IFN-γ.

2.3 Qualitative differences of $CD4^+$ $FoxP3^+$ regulatory T cells in SJL mice after *H. polygyrus* infection

H. polygyrus has previously been reported to expand the numbers of $Foxp3^+$ regulatory T cells in C57BL/6 and BALB/c mice (Finney *et al* 2007; Rausch *et al* 2008) and in particular to stimulate a significant rise in CD103 expression within the

Foxp3$^+$ T reg compartment (Finney *et al* 2007). The induction of CD103 is considered an activation marker for Tregs (Huehn *et al* 2004) and is strongly TGF-β dependent in *H. polygyrus*-infected mice (Reynolds and Maizels 2012). Furthermore, interfering with TGF-β signalling in chronically infected mice has been shown to increase worm expulsion (Grainger *et al* 2010), although depletion of Tregs in Foxp3-diphtheria toxin receptor mice did not alter the worm burden at day 14 (Rausch *et al* 2009).

Since the most susceptible strains of mice had higher levels of IFN-γ in response to *H. polygyrus*, and this has been linked to a lack of regulation in this system, regulatory T cell responses were assessed at day 7 post-infection in the 4 strains of mice. Perhaps surprisingly, the strain which exhibited the clearest increment in the percentage of MLN Foxp3$^+$CD4$^+$ Tregs, as measured by flow cytometry, was the most resistant SJL (Fig. 2.3 A). However, when this subset was further analysed for expression of CD103, it was notable that the SJL mouse showed much lower levels in the steady-state uninfected lymph nodes (as independently reported elsewhere (Tucker *et al* 2011)), and did not upregulate CD103 on infection in the same manner as BALB/c mice (Fig. 2.3 B). Similarly, fewer SJL Tregs upregulated GATA3 in response to infection (Fig. 2.3 C), which may be important, as GATA3 has been found to be required for functional Treg suppression in the gut (Wohlfert *et al* 2011). These data argue that although there is no correlation between susceptibility and the overall numbers of Foxp3$^+$ Tregs, a qualitative distinction may exist in the suppressive capacity of these cells in the most resistant SJL genotype.

Because CD103 induction is associated with TGF-β stimulation, and the known importance of TGF-β in *de novo* conversion of peripheral T cells to a regulatory phenotype, the ability of SJL and C57BL/6 T cells to convert from Foxp3$^-$ to Foxp3$^+$ T cells upon incubation with mammalian TGF-β, or with HES which contains a functional mimic of TGF-β (Grainger *et al* 2010), was measured. Both C57BL/6 and SJL T cells were able to upregulate Foxp3 expression, although the proportional increase was less marked in cells from the resistant SJL strain (Fig 2.3 D).

2.4 SJL mice have stronger and more wide-ranging antibody responses, although display a defect in IgE production, in response to *H. polygyrus* infection

Parasite-specific IgG1 antibodies are known to be protective against *H. polygyrus* infection (Pritchard *et al* 1983; Williams and Behnke 1983; McCoy *et al* 2008; Wojciechowski *et al* 2009; Liu *et al* 2010b), and their titre correlates with genetic resistance to this parasite (Ben-Smith *et al* 1999). As most serum antibodies are directed against antigens in the secreted HES products (Hewitson *et al* 2011a), anti-HES antibody levels were assayed. The resistant SJL mice were found to have 10-fold higher IgG1 titres than other strains (Fig 2.4 A), with a broader repertoire of total antibody:antigen recognition (Fig 2.4 B). Surprisingly, specific IgE production was significantly higher in the most susceptible strain, CBA (Fig 2.4 C) and relatively low, as previously noted (Ben-Smith *et al* 2003), in resistant SJL mice. No difference in anti-HES IgA titers was observed between strains (Fig 2.4 D). *H. polygyrus* infection also stimulates hyper-IgG and -IgE serum levels reflecting polyclonal antibody stimulation (Chapman *et al* 1979; Pritchard *et al* 1983; Urban *et al* 1991a). While total serum IgG1 levels rose more than ten-fold in infected mice of all strains (Fig 2.4 E), increased total IgE was only seen in the more susceptible genotypes, with SJL mice recording almost no increment in serum IgE concentrations (Fig 2.4 F). Overall, these data reinforce the conclusion that IgG1, rather than IgE or IgA, is the critical isotype required for immunity to *H. polygyrus* (McCoy *et al* 2008).

2.5 Levels of innate cytokines and innate lymphoid cells correlate with more resistance phenotypes in *H. polygyrus* infection

ILC2s have been identified as key instigators of Th2 responses, releasing IL-5 and IL-13 in response to the epithelial-derived cytokines IL-25, IL-33 and TSLP (Saenz *et al* 2008; Saenz *et al* 2010a; Oliphant *et al* 2011; Spits and Cupedo 2012). Their importance in regulation of commensal bacteria, balancing of inflammation and wound repair, and responses to infections in the gut (Sonnenberg and Artis 2012; Tait Wojno and Artis 2012), and in particular, in mediating protective responses to

intestinal helminths such as *N. brasiliensis* (Neill *et al* 2010) and *T. muris* (Saenz *et al* 2010b), has been demonstrated.

ILC responses were therefore evaluated, and it was found that IL-5$^+$ and IL-13$^+$ lineage-negative cells (negative staining for CD3, CD4, CD8α, CD19, CD11b, CD11c, F4/80, Gr1, DX5, MHCII) were induced in the MLN at day 7 post-infection with *H. polygyrus,* with higher levels of cells expressing cytokines in the most resistant strains (Fig 2.5 A, B). When expressed as a proportion of total MLN cells, 0.1-0.2% MLN cells from SJL mice were IL-5 producing ILCs (Fig 2.5 A), which was significantly higher than in BALB/c and C57BL/6 mice. Staining for IL-13 in ILCs was more variable (Fig 2.5 B, D).

The total numbers of eosinophils (CD11b$^+$ SiglecF$^+$) in the PL at day 7 post-infection correlate with the levels of IL-5$^+$ ILCs, which have been shown to promote eosinophils in other helminth models (Yasuda *et al* 2012; Molofsky *et al* 2013).

2.6 Expression of markers of alternatively activated macrophages correlates with resistance to *H. polygyrus*

Many helminth infections drive a specialized functional program in macrophages termed alternative activation, associated with expression of distinct gene products including Arg1, Chitinase-3-like protein 3 (also known as Ym-1), and RELM-α (otherwise FIZZ-1) (Kreider *et al* 2007). Total numbers of macrophages (CD11b$^+$ F4/80$^+$) in the PL, the site adjacent to parasite-induced intestinal inflammation, were highest in the most resistant SJL and BALB/c mice (Fig 2.6 A). Markers of alternative activation were strikingly elevated among these expanded peritoneal macrophage populations in the SJL and BALB/c strains, in which 15-25% expressed RELM-α and Ym1 within 7 days of infection (Fig. 2.6 B). These gene products were also upregulated in intestinal tissue, as found for RELM-α and Ym1 protein by ELISA (Fig. 2.6 C, D) and RELM-α by RT-PCR (Fig. 2.6 E). Similarly Arg1 mRNA levels were most upregulated from naïve levels in the SJL strain (Fig. 2.6 F). Upregulation of RELM-β expression, which has been reported to exert a direct anti-

parasite effect on *H. polygyrus* (Herbert *et al* 2009), was also maximal in infected SJL mice, although in this case CBA naïve and infected samples had very high levels of mRNA for this protein (Fig. 2.6 G).

2.7 Primary resistance to *H. polygyrus* infection is associated with granuloma formation and intestinal expression of Ym1

A notable feature of *H. polygyrus* infection in the more resistant animals is the development of numerous macroscopic granulomas in the gut wall by day 14 (Fig. 2.1E, H). Similar granuloma-like structures have been reported following secondary challenge of susceptible mice that have been cleared of primary infection by curative drug treatment (Liu *et al* 2004; Anthony *et al* 2006; Anthony *et al* 2007), as well as in resistant mice within the course of primary infection itself (Prowse *et al* 1979); these granulomas have been reported to be macrophage-rich with a significant influx of neutrophilic granulocytes (Morimoto *et al* 2004). Histological analysis of the duodenal wall showed that at day 14 post-infection, some granulomas in SJL mice still contained parasites although the great majority had emerged into the lumen (Fig. 2.7A). At this time point, all parasites in the other strains were found in the lumen.

To assess the level of alternative activation *in situ,* sections of gut wall (containing granulomas in the appropriate strains) were probed with antibody to the Chi3L3 (Ym1) protein product. As also illustrated in Fig. 2.7 B, SJL mice showed high levels of Ym1 protein both in and around the granuloma, often associated with large mononuclear cells (Fig. 2.7 D arrows). No significant staining was observed in the two more susceptible strains, or in gut tissues taken from uninfected mice of any genotype (data not shown).

2.8 Immunity to *H. polygyrus* is completely dependent on IL-4Rα-mediated signalling but is not significantly influenced by CD8 T cells and IFN-γ

It is well established that resistance to gastrointestinal nematodes requires IL4R-mediated signalling in both hematopoietic and non-hematopoietic cells (Urban *et al*

1998; Urban *et al* 2001). To test whether granuloma development was similarly dependent, BALB/c and congenic IL-4Rα-deficient mice were infected. Very clearly, no granulomas developed in the IL-4Rα$^{-/-}$ animals (Fig 2.8 A) and, as expected, IL-4Rα-deficient mice were highly susceptible to infection with increased fecundity per worm (Fig 2.8 B); they also failed to expel adult parasites by day 28 (Fig 2.8 C).
In the susceptible backgrounds, it was assessed whether high levels of IFN-γ expression within the CD4^{+} and CD8^{+} T cell subsets were responsible for their failure to expel *H. polygyrus*, as depletion of this cytokine from *T. muris*-infected mice is sufficient to confer resistance in normally susceptible animals (Else *et al* 1994). First, CD8^{+} T cells were depleted from C57BL/6 mice with monoclonal antibody YTS169; this resulted in modest reductions in egg counts, which did not attain statistical significance, while adult worm numbers were unchanged at day 28 (Fig 2.8 D, E).

Secondly, IFN-γ-deficient mice on the C57BL/6 background were infected with *H. polygyrus*: this genotype shows reduced worm loads at 28 days post-infection (Reynolds and Maizels 2012), but at the earlier time point of 14 days, no difference was observed in adult worm numbers (Fig 2.8 F). Unlike other experiments (Reynolds and Maizels 2012), egg production at day 14 was also unaltered (Fig 2.8 G), indicating that the effect of IFN-γ-deficiency is not profound in the C57BL/6 setting. Hence, IFN-γ contributes to susceptibility but in contrast to the *T. muris* system, does not fully account for failure of resistance in the susceptible mice.

2.9 Clodronate depletion of macrophages in BALB/c and SJL mice significantly compromises granuloma formation and immunity during *H. polygyrus* infection

As macrophages have been shown to be a major cell type in local inflammatory responses to *H. polygyrus* (Figs 6 and 7, and (Anthony *et al* 2007)), clodronate liposome administration was used to deplete phagocytic immune cells *in vivo*. Intravenous administration of liposomes containing the chemical clodronate, leads to internalization by, and specific toxicity to, macrophages and circulating monocytes

which subsequently undergo apoptosis (van Rooijen and Hendrikx 2010).

In BALB/c mice, clodronate treatment significantly impaired immunity as shown by increased adult worm (Fig. 2.9 A) and egg (Fig. 2.9 B) numbers at day 28. At this later time point, few granulomas were present in clodronate-treated mice (Fig. 2.9 C). Similarly, clodronate-treated SJL mice formed a reduced number of intestinal granulomas (Fig. 2.9 D), but continued to repress egg production (Fig 2.9 E), while adult worm expulsion was only slightly delayed (Fig. 2.9 F). Hence the strength of the contribution of clodronate-sensitive phagocytes to protection against helminth infection depends on the genetic background of the host. Clodronate treatment was shown to reduce expression of CD115 on the surface of $CD11b^+$ cells in the blood, and the proportion of $CD11b^+$ cells by ~60-70% (Fig 2.9 G, H). Therefore, clodronate treatment does not completely deplete macrophages and monocytes from the circulation. Given the significant effects of the treatment however, it is clear that these cells contribute directly to the composition of the granuloma and to anti-parasite immunity.

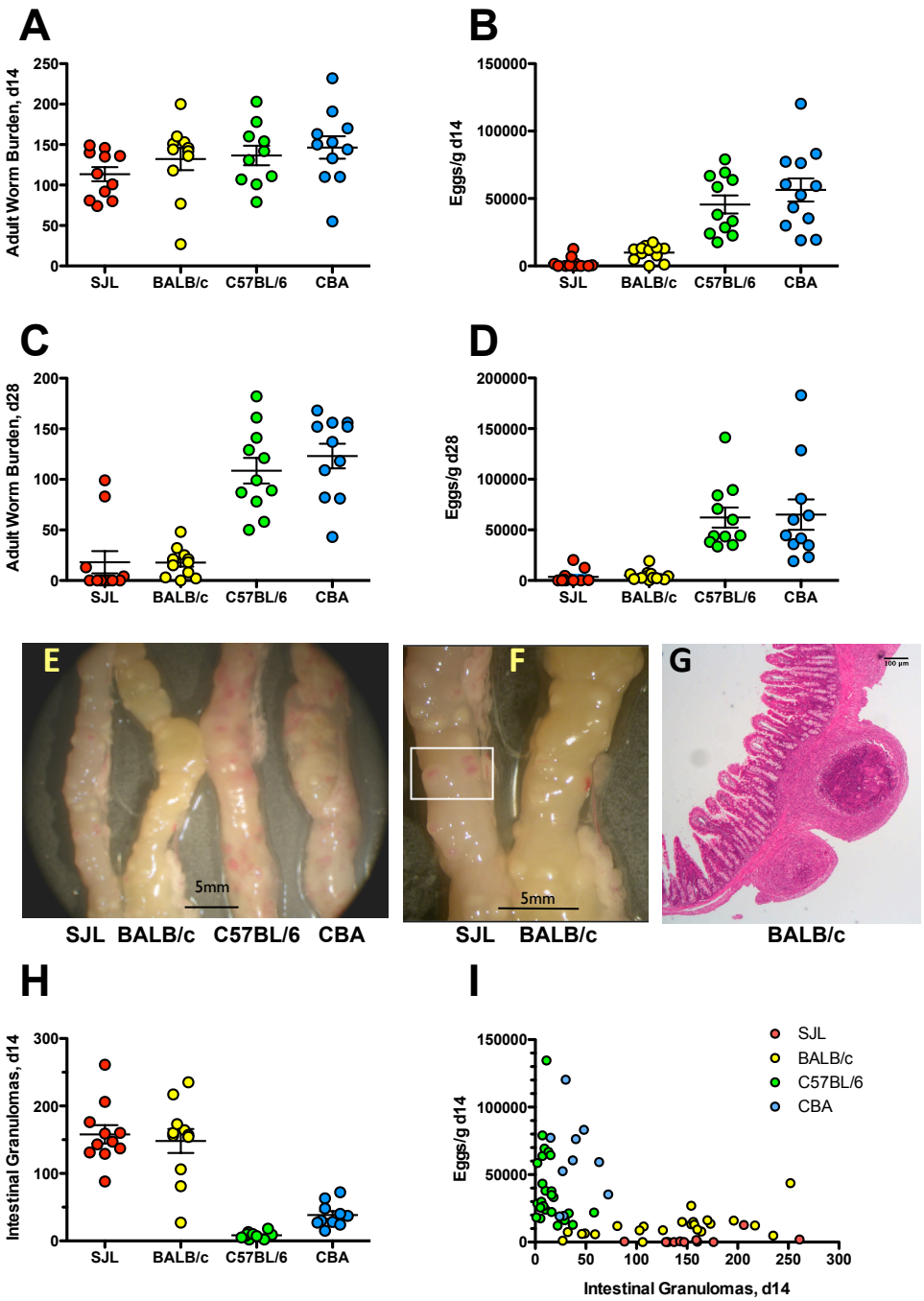


Figure 2.1 - Variation in susceptibility to primary infection with *H. polygyrus* manifests first with early differences in parasite fecundity and granuloma formation, and subsequent loss of adult worms.

Age-matched female SJL, BALB/c, C57BL/6 and CBA mice were infected with 200 *H. polygyrus* L3 larvae by gavage. Data presented are pooled from two independent experiments. Bars indicate mean and standard error of the mean.
Luminal adult parasites (A, B) and faecal egg counts (C, D) were enumerated at days 14 and 28 post-infection. Representative images of d14 intestinal granulomas in different mouse strains were taken at x0.75 (E) and x1.25 (F) magnifications, and from a d14 infected BALB/c mouse, hemotoxylin and eosin stained (G). Image taken at x4 magnification.
The number of granulomas in the small intestine were enumerated at day 14 of infection (H) and the negative relationship between egg numbers and granulomas at day 14 of infection is presented (I).

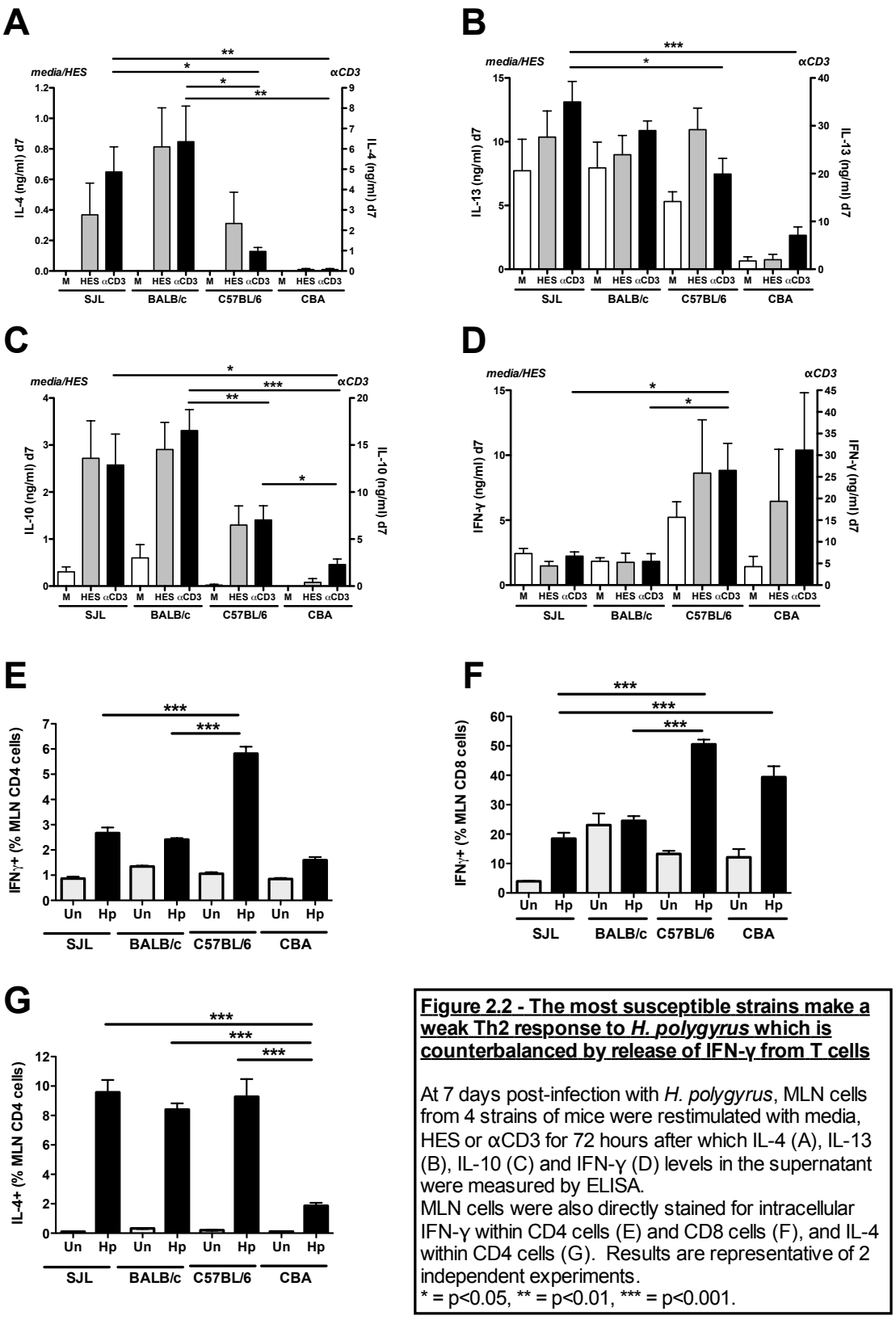


Figure 2.2 - The most susceptible strains make a weak Th2 response to *H. polygyrus* which is counterbalanced by release of IFN-γ from T cells

At 7 days post-infection with *H. polygyrus*, MLN cells from 4 strains of mice were restimulated with media, HES or αCD3 for 72 hours after which IL-4 (A), IL-13 (B), IL-10 (C) and IFN-γ (D) levels in the supernatant were measured by ELISA.
MLN cells were also directly stained for intracellular IFN-γ within CD4 cells (E) and CD8 cells (F), and IL-4 within CD4 cells (G). Results are representative of 2 independent experiments.
* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

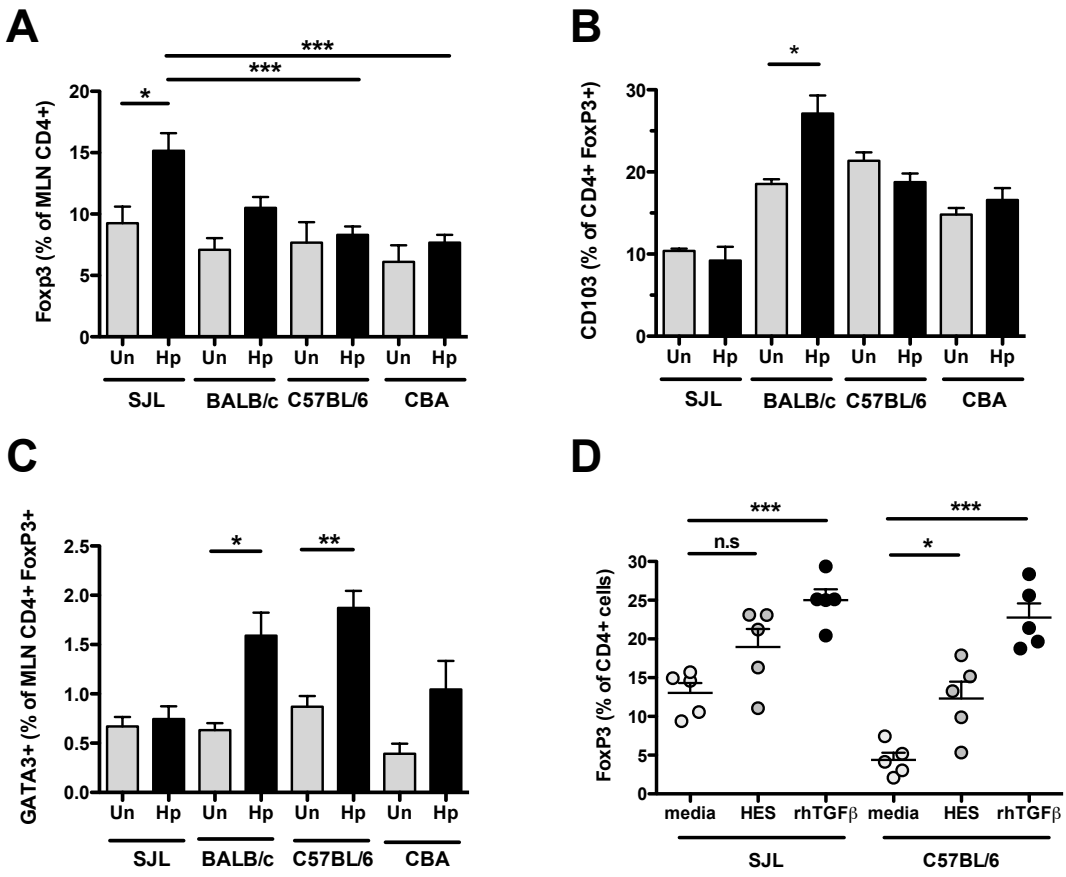


Figure 2.3 - Qualitative differences of CD4$^+$ FoxP3$^+$ regulatory T cells in SJL mice after *H. polygyrus* infection

CD4$^+$ T cells from naïve or day 7 *H. polygyrus*-infected female SJL, BALB/c, C57BL/6 and CBA mice were stained for expression of Foxp3 (A) together with CD103 (B), GATA3 (C). Results are pooled from 2 experiments. Positive cells are expressed as the mean with standard error from 2-6 animals per group.
CD4$^+$ splenocytes were separated and cultured for 72 hours with media, 10µg/ml HES or 10ng/ml rhTGFβ, and FoxP3 expression assessed (D).
* = p<0.05, ** = p<0.01, *** = p<0.001

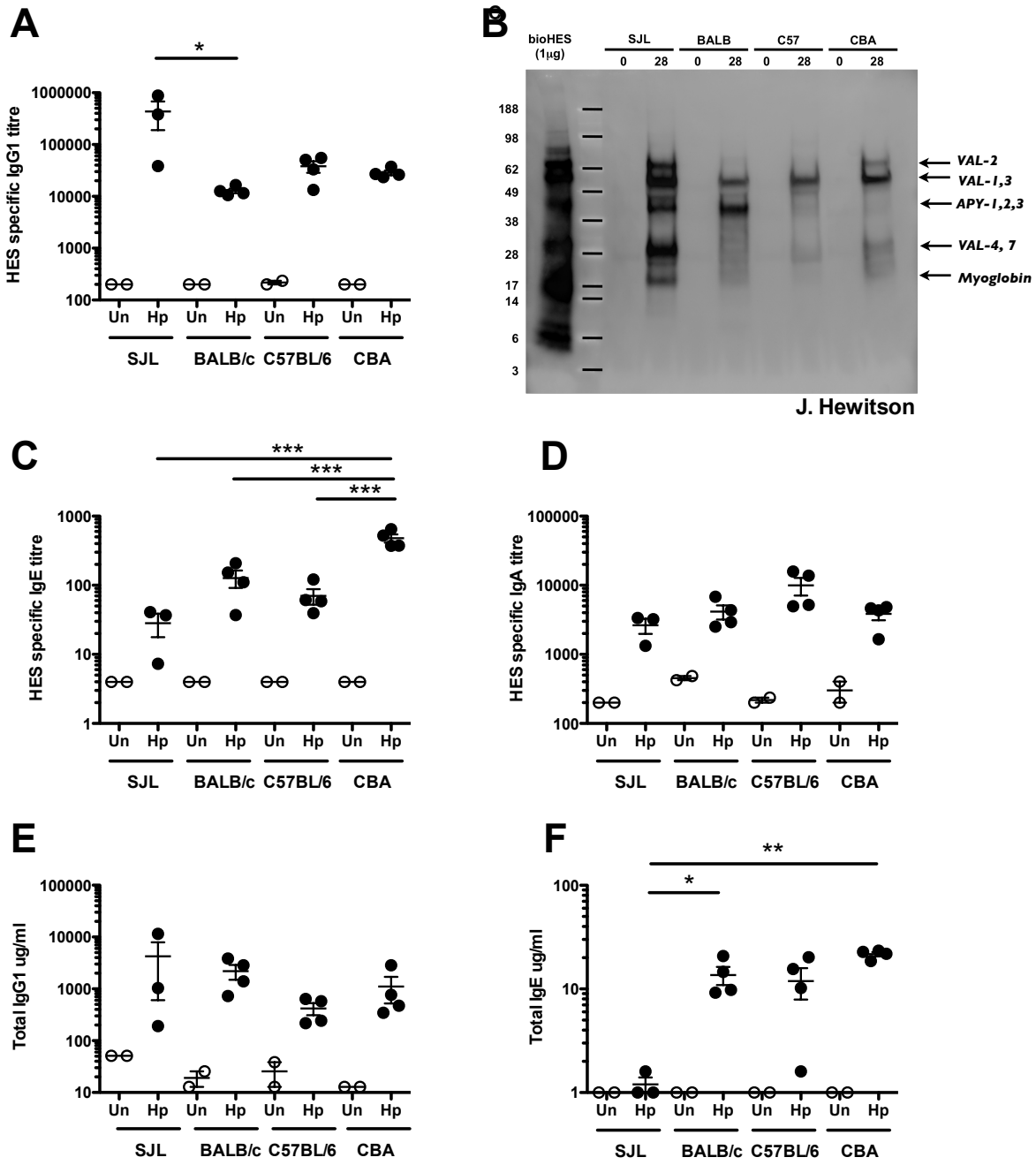


Figure 2.4 - SJL mice have stronger and more wide ranging antibody responses, although display a defect in IgE production in response to *H. polygyrus* infection

Anti-HES IgG1 (A), IgE (C) and IgA (D) serum antibodies from day 28-infected mice were measured by ELISA and endpoint titres determined. Specificity of serum antibodies of naive and 28-day infected mice was profiled by immunoprecipitation of biotinylated HES followed by binding of enzyme-linked streptavidin (experiment carried out by Dr James Hewitson) Positions of major antigens are indicated. (B). Total serum IgG1 (E) and IgE (F) levels in naïve and 28-day infected mice of each strain were detarmined by ELISA aagainst a standard curve of recombinant mouse IgG1 or IgE.
Mean values with standard errors are presented; data are representative of several replicate experiments.
* = p< 0.05, ** = p<0.01, *** = p<0.001

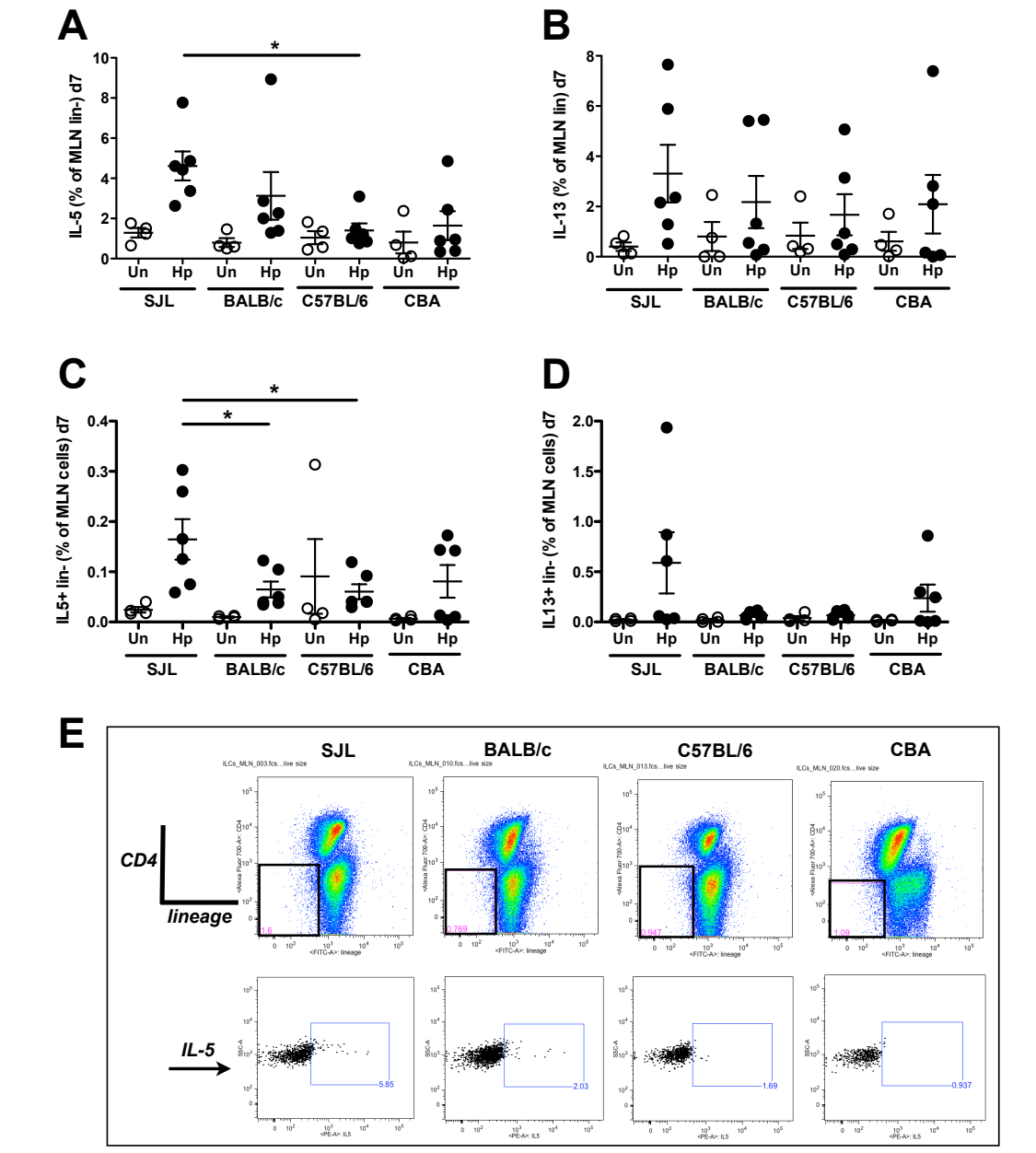


Figure 2.5 - Levels of innate cytokines and innate lymphoid cells correlate with more resistant phenotypes in *H. polygyrus* infection

At 7 days post-infection with *H. polygyrus* the proportion of lineage negative cells expressing IL-5 (A) and IL-13 (B) were analysed, also expressed as proportions of all MLN cells (C, D). Representative FACS plots of lineage negative gating and IL-5 staining withiin this population (E).
The total number of eosinophils (CD11b+ SiglecF+) in the PL of naive and day 7-infected mice was enumerated (F).
Data are from 2 experiments combined. * = p<0.05

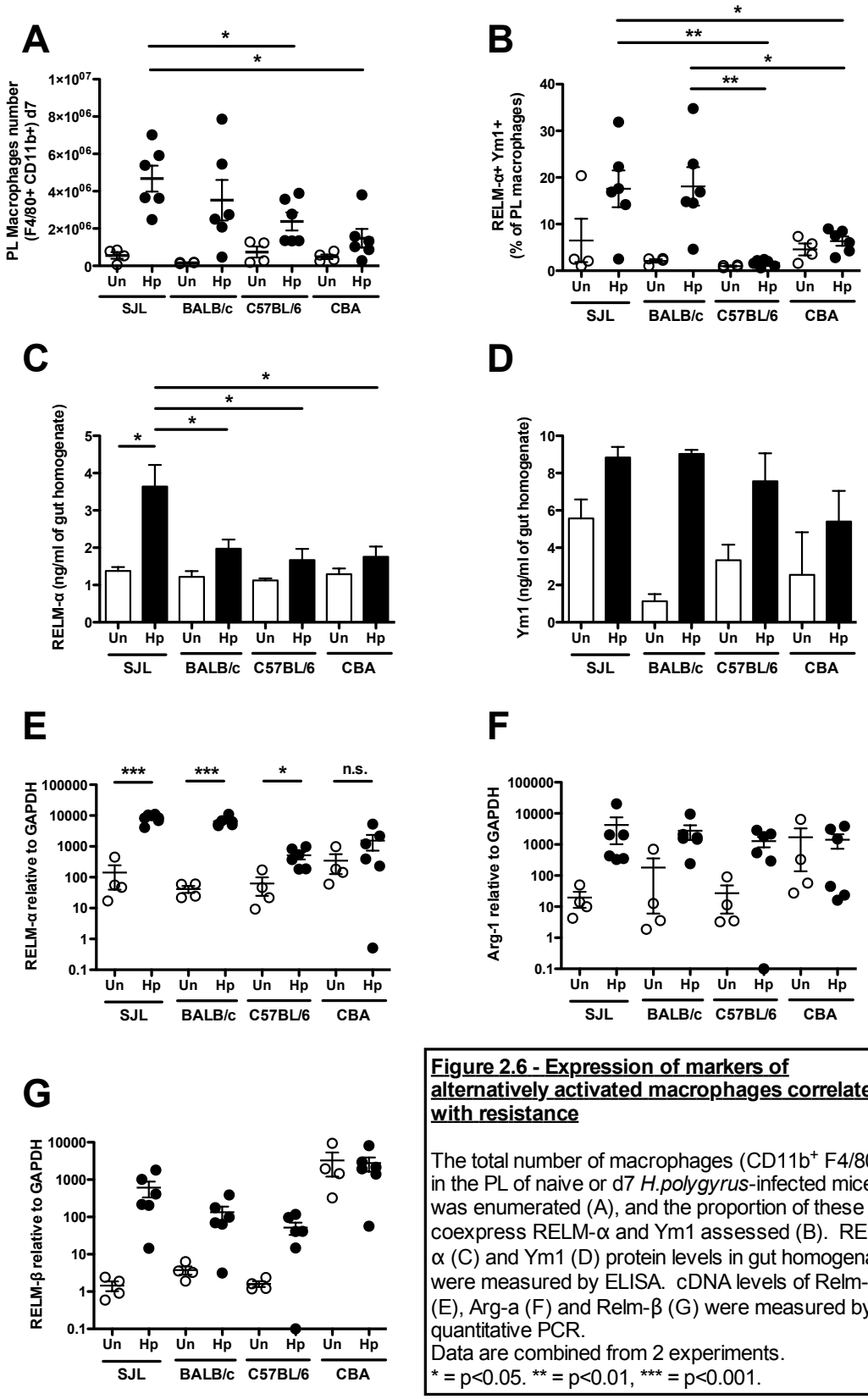


Figure 2.6 - Expression of markers of alternatively activated macrophages correlates with resistance

The total number of macrophages ($CD11b^+$ $F4/80^+$) in the PL of naive or d7 *H.polygyrus*-infected mice was enumerated (A), and the proportion of these to coexpress RELM-α and Ym1 assessed (B). RELM-α (C) and Ym1 (D) protein levels in gut homogenate were measured by ELISA. cDNA levels of Relm-α (E), Arg-a (F) and Relm-β (G) were measured by quantitative PCR.
Data are combined from 2 experiments.
* = $p<0.05$. ** = $p<0.01$, *** = $p<0.001$.

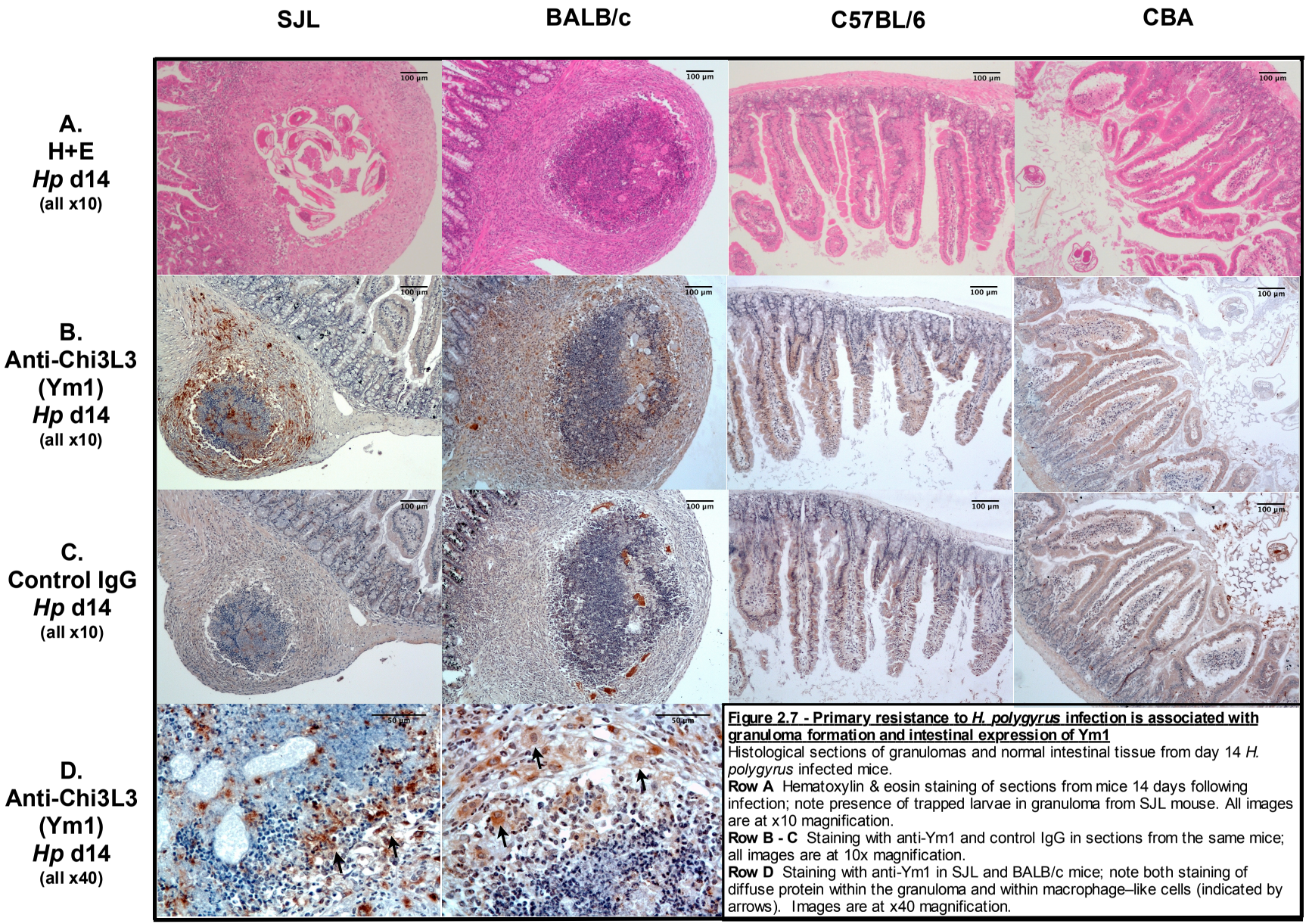


Figure 2.7 - Primary resistance to *H. polygyrus* infection is associated with granuloma formation and intestinal expression of Ym1

Histological sections of granulomas and normal intestinal tissue from day 14 *H. polygyrus* infected mice.

Row A Hematoxylin & eosin staining of sections from mice 14 days following infection; note presence of trapped larvae in granuloma from SJL mouse. All images are at x10 magnification.

Row B - C Staining with anti-Ym1 and control IgG in sections from the same mice; all images are at 10x magnification.

Row D Staining with anti-Ym1 in SJL and BALB/c mice; note both staining of diffuse protein within the granuloma and within macrophage–like cells (indicated by arrows). Images are at x40 magnification.

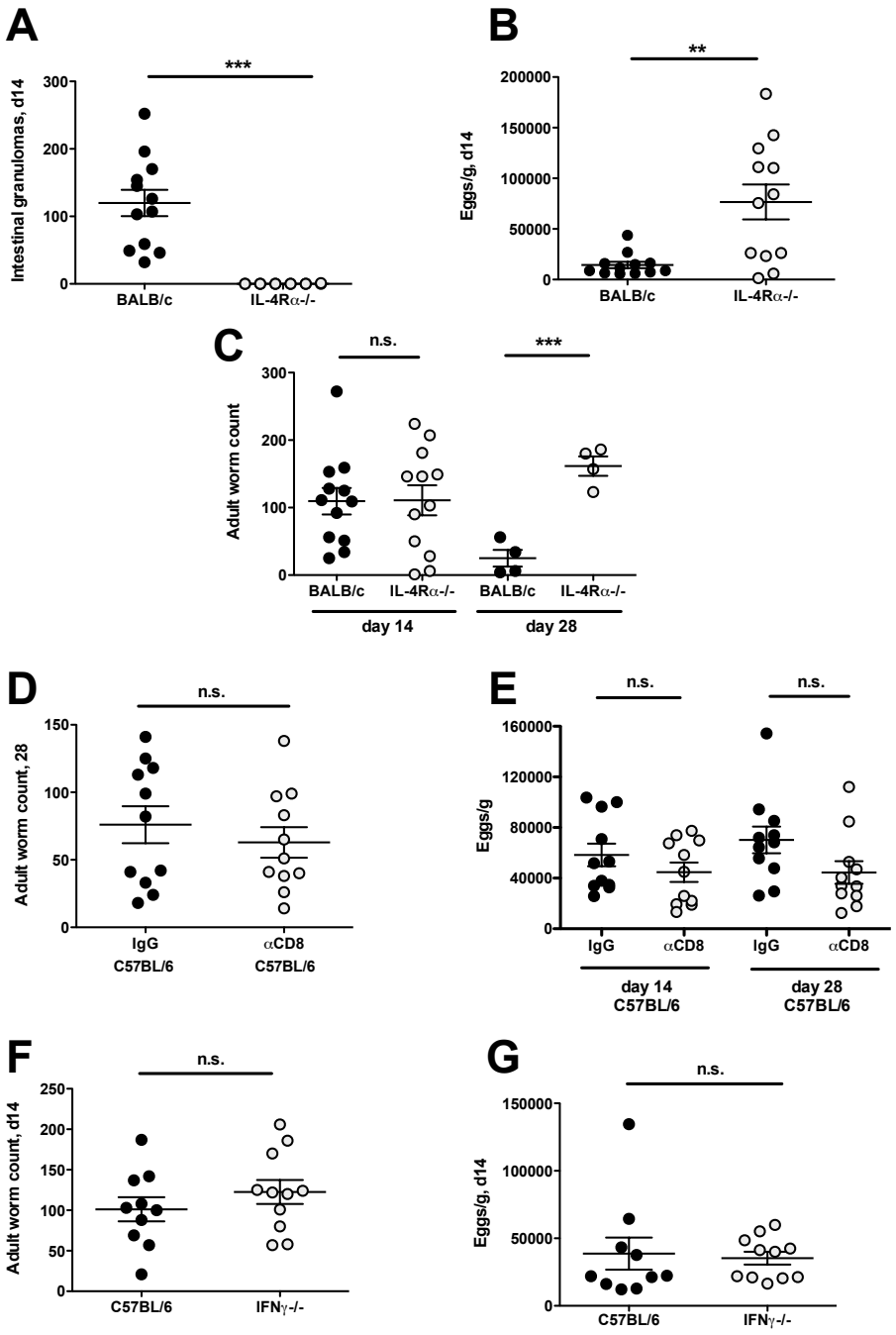


Figure 2.8 - Immunity to *H. polygyrus* is completely dependent on IL-4Rα-mediated signaling but is not significantly influenced by CD8 T cells and IFN-γ

BALB/c and IL4-Rα$^{-/-}$ mice were infected with *H. polygyrus* and at day 14 post-infection intestinal granulomas (A) and faecal egg counts (B) were enumerated. Adult worm counts were taken at days 14 and 28 post-infection (C).
C57BL/6 mice were treated with αCD8 or control IgG during *H. polygyrus* infection, with adult worm counts enumerated at day 14 post-infection (D), and faecal egg counts at days 14 and 28 (E).
C57BL/6 and IFN-γ$^{-/-}$ mice were infected with H. *polygyrus* and adult worms (F) and faecal egg counts (G) enumerated at day 14 post-infection.
All graphs represent 2 experiments combined, with the exception of d28 worm burden data in Figure C, which is data from one experiment. ** = $p<0.01$, *** = $p<0.001$

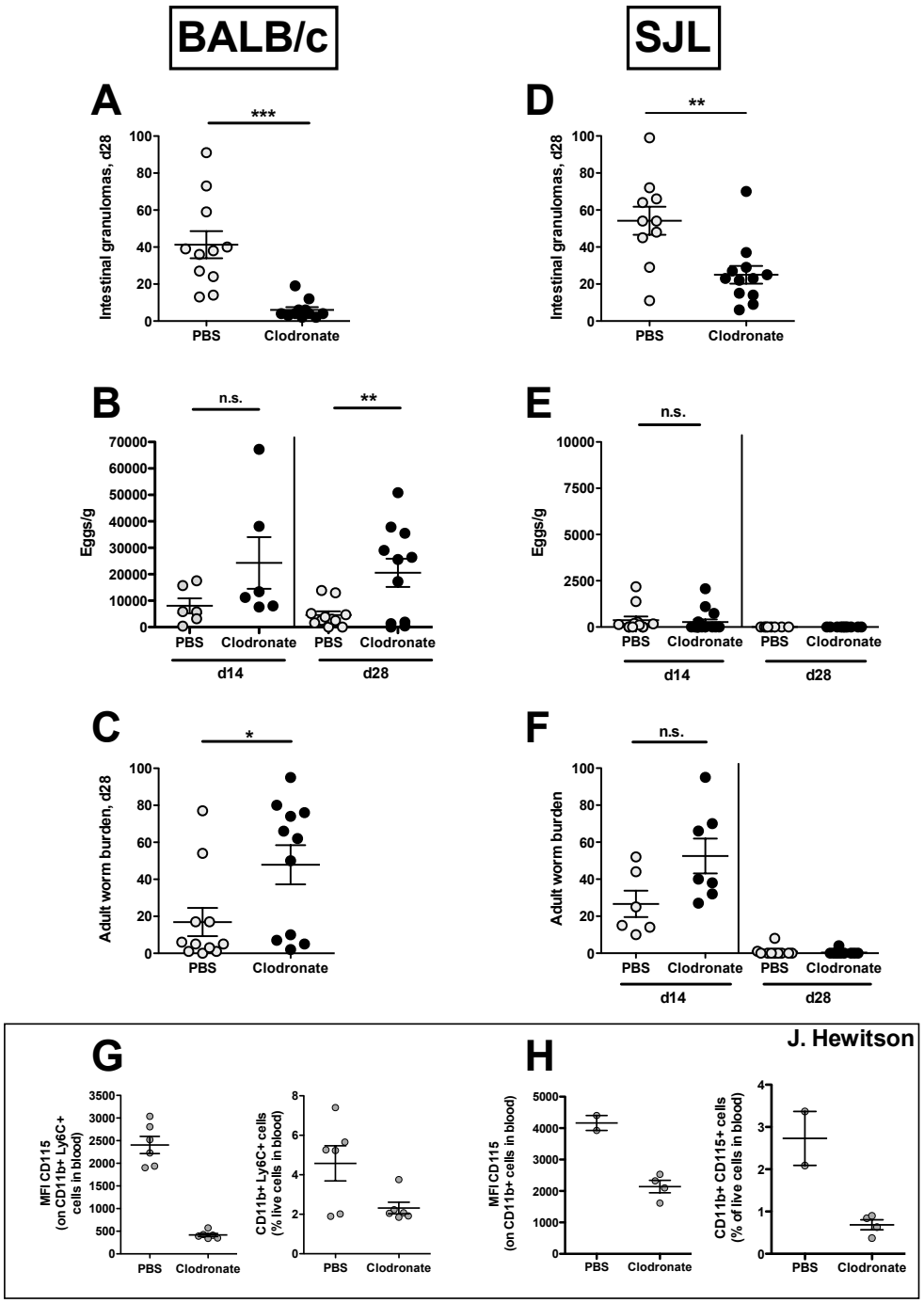


Figure 2. 9 - Clodronate depletion of macrophages in BALB/c and SJL mice has significant effects on granuloma formation and immunity during *H. polygyrus* infection

BALB/c and SJL mice were treated with clodronate or PBS i.v. during *H. polygyrus* infection, and day 28 intestinal granulomas (A, D), day 14 and 28 faecal egg burdens (B, E) and adult worms (C, F) were enumerated. All results are pooled from 2 experiments and bars represent the mean and standard error. * = p< 0.05, ** = p<0.01, *** = p<0.001
The MFI of CD115 on CD11b+ cells and the proportion of CD11b+ cells in the blood of mice treated with PBS or clodronate were assessed for BALB/c (G) and SJL mice (H), from one experiment each (experiment carried out by Dr James Hewitson).

Discussion

Immunity to gastrointestinal helminth infections requires the appropriate and co-ordinate responsiveness of the innate and adaptive immune systems (Anthony *et al* 2007; Neill and McKenzie 2011; Maizels *et al* 2012). The degree to which immunity successfully expels the parasite varies, however, according to the genetic status of the host; thus, comparisons of genetically susceptible and resistant genotypes can identify key components and mediators that are required for most effective immune protection. Different mouse strains show diverse patterns of susceptibility to *H. polygyrus* infection (Enriquez *et al* 1988; Wahid and Behnke 1993b), with resistance clearly associated with strength of Th2 responsiveness (Wahid and Behnke 1993a; Lawrence and Pritchard 1994).

By day 14 post-infection with *H. polygyrus*, dramatic differences were apparent in worm fecundity between 4 strains of mice, which preceded subsequent worm explusion in the resistant genotypes. Those strains able to limit egg production and curtail infection displayed a suite of enhanced Type 2 responses, including ILC-derived cytokines, T cell production of IL-4, -10 and -13, eosinophilia and AAMΦ. Previous studies have highlighted such components in individual strains but these have not previously been correlated to the differential susceptibility of inbred mouse strains. The positive correlation between levels of the cardinal type 2 cytokines IL-4 and IL-13, and early expulsion of *H. polygyrus*, is entirely consistent with published reports on the strength of Th2 responses in resistant mice. More surprising however is that IL-10 not only parallels the major Th2 cytokines, but shows a more extreme polarisation: thus the more resistant strains express the highest IL-10 when judged by elevated intracellular cytokine staining and by antigen-specific recall responses *in vitro*. Poor, or slow, IL-10 production could permit higher IFN-γ levels among both $CD4^+$ and $CD8^+$ T cells in the more susceptible C57BL/6 and CBA mice which feed back to dampen the Th2 response more broadly. An interesting possibility is that IL-10 acts in mice in a manner similar to that in helminth-infected humans, promoting an IgG isotype (IgG1 in mice, IgG4 in humans) while suppressing IgE (Satoguina *et al* 2005).

Perhaps the most striking feature of the BALB/c and SJL strains is the extensive number of macrophage-rich granulomas in the intestinal wall which (as shown in SJL mice) envelop the larval stage of the parasite. Such granulomas appear to be similar to those described in secondary infection, in which it was suggested that AAMΦ in the granulomas played a key role in mediating parasite killing (Anthony *et al* 2006; Anthony *et al* 2007; Patel *et al* 2009); the granulomas have also been reported to be somewhat dependent on IL-21R signalling (Fröhlich *et al* 2007). Primary granulomas are similarly macrophage-dependent, and associated with alternative activation, as shown in a number of ways. Gene expression of AAMΦ markers Arg1 and RELM-α was maximal in the resistant SJL strain and immunohistochemical staining showed high levels of Ym1 protein in the granulomas of the most resistant strains. Secondly, granulomas were absent in the IL-4Rα-deficient setting, in which alternative activation of macrophages does not occur in response to helminths (Herbert *et al* 2004; Loke *et al* 2007). Furthermore, clodronate depletion of macrophages reduced granuloma formation and control of egg and worm burdens in BALB/c and SJL mice.

The experimental depletion of macrophages using clodronate is not entirely satisfactory due to the incomplete ablation, so that use of alternative methods would be desirable. Conditional gene targeting in myeloid cells, such as in the LysM-cre mouse (Clausen *et al* 1999), which has an insertion of Cre cDNA in the M lysozyme locus endogenous to myeloid cells, results in deletion of the targeted gene of choice in most mature macrophages, but is not complete, and also affects other granulocytes and a small percentage of DCs. This system has also been used with a diphtheria-toxin receptor (DTR) cassette insertion to create LysM-DTR mice, which upon DT administration display ablation of lysM expressing cells, although the range of cells depleted and efficiency of depletion has been varied (Hume 2011). Several other strategies for altering gene expression in macrophages, or deleting them completely, have been published (Hume 2011) and could be utilized to assess more specifically the role of macrophages in the granuloma and in immunity to *H. polygyrus*.

A further factor in the resistance of SJL mice may be a subtle defect in activation of regulatory T cells; this may not be evident solely in helminth infection, as the SJL mouse is also prone to a series of autoimmune conditions, and indeed defective T cell suppression has long been noted in this strain (Hutchings *et al* 1986). If SJL mice are deficient in inducible Tregs, this may allow them to more rapidly deploy AAMΦ, as it has recently been reported that mice lacking the conserved nucleotide sequence CNS-1 in the Foxp3 gene (which allows TGF-β-mediated signals to stabilize Treg function), and lacking inducible Tregs, display uncontrolled accumulation of Ym1-expressing AAMΦ (Josefowicz *et al* 2012). Future studies should explore the role not only of Tregs in this setting, but regulatory B cells which were previously found to be active in *H. polygyrus* infections of the more susceptible C57BL/6 strain (Wilson *et al* 2010).

These data illustrate the multi-faceted and complex nature of innate and adaptive immune mechanisms involved in the control of *H. polygyrus*, and shows the extra layer of complexity added by the genetic background of the host. This provides a useful platform for the design of future experiments with *H. polygyrus,* as immune mediated effects on parasitological parameters can be examined to maximal effect in the most suitable wild-type strain. Correlates of immunity discovered in this way could be extrapolated to human studies and used to predict responsiveness to helminth infections or treatment of them in different cohorts. Finally, future studies could utilize high throughput sequencing and gene array technology to more comprehensively assay patterns of host gene expression associated with resistance or susceptibility to helminth infections.

<u>Key Findings</u>

- Strains of mice more resistant to *H. polygyrus* have a higher frequency of intestinal granulomas, which show strong expression of Ym1, and are significantly depleted in number upon clodronate treatment of the mice, especially in BALB/c mice.

- In the most resistant strain, SJL, other mechanisms of immunity are likely to compensate for the loss of macrophages, as granulomas are not depleted to the same extent and worms are still expelled almost fully by day 28.
- The most susceptible strains of mice have weak Th2 responses, which are counterbalanced by release of high levels of IFN-γ from both $CD4^+$ and $CD8^+$ cells.
- Proportions of ILC2s in the MLN of infected mice are significantly higher in the most resistant strains of mice than in more susceptible strains – the induction of these cells has not previously been shown in *H. polygyrus* infection, and as they are thought to be the key initiators of Th2 responses, it will be important to investigate them further in this setting.

Chapter 3

The role of MIF in immunity to gastrointestinal helminths

Introduction

Immune responses to multicellular organisms, such as helminth parasites, are multifaceted and complex, including a plethora of cytokines, chemokines, cells and systems to control and clear the worms, and limit their spread further through the host population (Gause *et al* 2003; Anthony *et al* 2007; Allen and Maizels 2011; Maizels *et al* 2012).

To investigate further the mechanisms of resistance to *H. polygyrus*, the cytokine MIF was selected, as a link between the innate local inflammatory responses observed in the formation of granulomas, and the systemic adaptive responses observed later in infection (see Chapter 2). MIF is one of the oldest cytokines to have been described, when in the 1960s it was found to be involved in the delayed-type hypersensitivity response and the inhibition of cell activation and migration *in vitro* (David 1966). Since then, the highly pleiotropic nature of this molecule, in terms of expression and function, has been revealed.

Many cell types have been found to express MIF, and in most areas of the body, especially those at barrier surfaces, such as lung, skin, gastrointestinal tract and genito-urinary tracts (Calandra and Roger 2003). As well as epithelial cells (Maaser *et al* 2002), immune cells including T and B lymphocytes, macrophages, monocytes, DCs, neutrophils, eosinophils, mast cells and basophils have all been described as making MIF, with the unusual feature of being made constitutively, and stored in intracellular pools for instant release upon stimulation (Bernhagen *et al* 1998; Lue *et al* 2002; Calandra and Roger 2003). MIF is also expressed at high levels in organs involved in stress responses such as the brain, adrenal and pituitary glands (Calandra and Bucala 1997; Donnelly and Bucala 1997), and in tumours, where it inhibits tumour cell lysis by cytotoxic T cells and NK cells (Repp *et al* 2000; Abe *et al* 2001), and induces immune suppressive cell populations such as tumour associated

macrophages and MDSCs (Bach *et al* 2008; Simpson *et al* 2012; Yaddanapudi *et al* 2013). Some studies have shown a correlation between MIF expression and worsened cancer prognosis (Meyer-Siegler *et al* 2002; Bach *et al* 2008). There is also evidence that, although $MIF^{-/-}$ mice have viable offspring, the molecule has a role in foetal development, having been detected in multiple tissues during organogenesis (Kobayashi *et al* 1999; Shimizu *et al* 2005; Shen *et al* 2012).

The role of MIF in inflammation and in inflammatory diseases is well documented (Donnelly and Bucala 1997; Ohkawara *et al* 2005; Bach *et al* 2008; Nishihira 2012). MIF production is significantly increased in patients with ulcerative colitis (Murakami *et al* 2001) and a polymorphism in the MIF gene is associated with a genetic predisposition of humans to Crohn's disease (Griga *et al* 2007). In experimental models of inflammatory bowel disease in mice, MIF deficiency or administration of anti-MIF antibody ameliorates inflammatory symptoms (de Jong *et al* 2001; Ohkawara *et al* 2002). MIF is also important in other inflammatory diseases such as airway allergy (Wang *et al* 2006; Amano *et al* 2007; Magalhães *et al* 2007) atherosclerosis, arthritis, lupus, (Santos and Morand 2009), pancreatitis (Sakai *et al* 2003), cystic fibrosis (Adamali *et al* 2012), and atopic dermatitis (Shimizu *et al* 1997). The known MIF polymorphisms in humans cause systemic-onset juvenile arthritis (Donn *et al* 2001) and influence the severity of rheumatoid arthritis in a cohort of patients (Baugh *et al* 2002), correlate with susceptibility to chronic hepatitis B and associated liver cirrhosis (Zhang *et al* 2013), gestational diabetes mellitus and metabolic syndrome (Aslani *et al* 2011) and cystic fibrosis (Plant *et al* 2005). MIF is also strongly induced in healing wounds in humans and mice (Gilliver *et al* 2010), although its precise role is unclear. The role of MIF as a chemokine and activator of inflammatory cells could explain the findings that MIF-deficiency, or blocking, led to the slower migration of skin fibroblasts and keratinocytes to the wound site and therefore slower healing (Abe *et al* 2000; Zhao *et al* 2005; Dewor *et al* 2007).

MIF shows 90% identity between humans, mice, rats and cattle, and homologues are also found in birds, fish, plants and parasites (Bernhagen *et al* 1998; Calandra and Roger 2003). It is a 114-amino acid protein with a molecular mass of 12.5 kDa, and

has a homo-trimer molecular structure (Bucala 1996; Suzuki *et al* 1996). MIF has no signal sequence (Bernhagen *et al* 1994), and so is not exported through the endoplasmic reticulum. It is not part of any cytokine superfamily (Calandra and Roger 2003), but does share structural homology with several bacterial enzymes, and has been shown to have catalytic activity itself as a tautomerase (Bernhagen *et al* 1998; Swope *et al* 1998). However, whether this enzymatic activity is necessary for its *in vivo* biological functions is unclear, as when a tautomerase-null MIF gene was knocked-in to replace the native MIF gene in mice, the molecule still bound to its surface receptor CD74 and induced the normal intracellular signaling cascade (Fingerle-Rowson *et al* 2009), and several mutant recombinant MIF proteins still had the immunological effects as normal MIF (Bendrat *et al* 1997; Hermanowski-Vosatka *et al* 1999; Kleemann *et al* 2000).

The MIF receptors have been identified as CD74 and CXCR4, which form a homodimer (Shi *et al* 2006; Schwartz *et al* 2009; Lue *et al* 2011; Schwartz *et al* 2012), and CXCR2 (Bernhagen *et al* 2007; Weber *et al* 2008). Binding through CD74 activates the extracellular-signal-regulated kinase 1/2 (ERK1/ERK2) and mitogen-activated protein kinase (MAPK) signalling pathway which leads to cell proliferation, upregulation of TLR4 on the cell surface (Roger *et al* 2001) and sustained cell survival (by negatively regulating the apotosis-inducing factor p53 (Mitchell *et al* 2002) roles consistent with the inflammatory nature of MIF. MIF-deficient mice are susceptible to intracellular bacterial infection, and this is associated with reduced levels of inflammatory cytokines such as TNF, IFN-γ and IL-12 (Koebernick *et al* 2002). However, too high a level of MIF can exacerbate lethal endotoxic shock caused by LPS and *E. coli* (Bernhagen *et al* 1993). MIF is released from macrophages upon LPS stimulation and promotes the upregulation of pro-inflammatory mediators including Th1 cytokines and NO, and inhibits their migration, in an autocrine fashion (Calandra *et al* 1994; Bernhagen *et al* 1998). The role of MIF in Th2 responses has been less widely documented but evidence suggests that in a Th2 environment, such as a helminth infection, MIF can synergize with IL-4 to promote the alternative activation of macrophages (Prieto-Lafuente *et al* 2009). Indeed, MIF has been found to be crucial in inflammatory responses in both

cutaneous (Yoshihisa *et al* 2010; Das *et al* 2011) and airway allergy models (Magalhães *et al* 2007).

Due its involvement in some of the most debilitating inflammatory autoimmune disease in humans, efforts to target MIF in therapy are ongoing (Bucala 2013). To this end several classes of MIF inhibitor are available (Winner *et al* 2008; Garai and Lóránd 2009; Xu *et al* 2013), as are anti-MIF antibodies, and recently a novel tool to knock down MIF specifically in APCs, via a Dectin-1-specific targeting of antisense MIF oligonucleotides into the cells, proved successful in a model of IBD (Nishihira 2012).

In terms of parasitic diseases, and more specifically, immunity to helminth infections, MIF has been less well characterized. In experimental *S. mansoni* infection, MIF was found to be secreted by intact egg granulomas *in vitro* (Boros *et al* 1973) and to act as a chemoattractant for eosinophils to the granulomas in the gut and liver, although MIF deficiency had no effect on worm, egg or granuloma numbers (Magalhães *et al* 2009). MIF mRNA was also detected in the adrenal and pituitary glands of baboons that were experimentally infected with *S. mansoni,* and was hypothesised by the authors to regulate hormone secretion in this setting (Morales-Montor *et al* 2003). Interestingly, MIF is also involved in the response of the intermediate snail host to infection with *S. mansoni* (Baeza Garcia *et al* 2010). Neutralising MIF in mice with rabbit anti-MIF at the time of liver granuloma formation and parasite egg release, compromised immunity to *S. japonicum,* which corresponded with decreases in TNF-α and IL-10 (Stavitsky *et al* 2003). The only other helminth parasite that has been studied with relation to MIF is *Taenia crassiceps*, to which $MIF^{-/-}$ mice are highly susceptible (Rodríguez-Sosa *et al* 2003). Susceptibility in these mice was not due to a failure in Th1 or Th2 cytokine production, but was associated with downregulated macrophage responses in the peritoneal cavity.

There are several more publications on the role of MIF in protozoan parasites where data is often conflicting, due to the many different strains of parasite, and variable host immune environment and genetic background (de Dios Rosado and Rodríguez-Sosa 2011; Bozza *et al* 2012). Generally, MIF is essential in experimental

Toxoplasmosis for the necessary Th1 response and classical macrophage activation needed to control the parasite burden (Flores *et al* 2008; Terrazas *et al* 2010b) but this can come with the cost of increased inflammation and tissue pathology (Cavalcanti *et al* 2011). MIF has also been shown to directly activate macrophages to kill *Leishmania* parasites through upregulation of TNF-α and iNOS (Juttner *et al* 1998), and furthermore, $MIF^{-/-}$ mice are highly susceptible to *L. major* infection, which is associated with lower levels of NO, not a defect in Th1 versus Th2 responses (Satoskar *et al* 2001). The same is true for *Trypanosoma* infections, in which MIF deficiency rendered mice susceptible to high parasitemia and severe immunopathology in the heart and skeletal muscle, associated with reduced levels of proinflammatory cytokines (Reyes *et al* 2006). High serum levels of MIF also correlate with more severe Chagas-related heart disease in humans (Cutrullis *et al* 2013).

Studies into malaria have more conflicting outcomes. It is clear that MIF is a risk factor for mortality in patients with cerebral malaria, caused by *Plasmodium falciparum* (Clark *et al* 2003; Jain *et al* 2009). Some studies have shown that children with a history of severe *P. falciparum* infections have lower levels of transcript and circulating MIF, than those with repeated mild infections (Awandare *et al* 2006; Awandare *et al* 2007), indicating that a propensity to make MIF is protective in childhood malaria. However, MIF seems to be associated with susceptibility and mortality in *P. chabaudi* infection in mice (Malu *et al* 2011) and associated with severe anaemia in this system (McDevitt *et al* 2006).

Homologues of MIF are widely documented in parasitic helminths (Vermeire *et al* 2008), and have been shown to contribute to the immune responses of the hosts they inhabit (Falcone *et al* 2001; Prieto-Lafuente *et al* 2009; Younis *et al* 2011). MIF is also found in the secretions of *H. polygyrus* (Hewitson *et al* 2011b). As MIF has been found to be important in development, it may be that its presence in organisms that would otherwise act to evade inflammatory immune responses, is explained by this phenomenon. Moreover, helminth MIF homologues have been found to downregulate bystander Th2 responses (Park *et al* 2012), and so may directly act to dampen anti-helminth immunity in this way.

As the contribution of MIF to the immune responses towards helminths has not been fully investigated, experiments were undertaken, primarily utilizing the $MIF^{-/-}$ mouse (Bozza *et al* 1999), to more extensively characterize the influence of MIF in cellular and molecular immunity to *H. polygyrus*, and the more acute gastrointestinal helminth *N. brasiliensis*.

Results

3.1 MIF deficiency renders mice more susceptible to *H. polygyrus* than BALB/c wild-type mice

To test the immunological role of MIF in helminth infection, mice with transgenic null alleles at this locus, backcrossed onto a BALB/c background, were infected with *H. polygyrus*. At day 14 of primary infection, $MIF^{-/-}$ mice harboured the same number of adult parasites as BALB/c wild-type controls (Fig 3.1 A). At this time-point the mean egg count in $MIF^{-/-}$ mice was 60% higher than BALB/c controls, although the difference did not reach statistical significance (Fig 3.1 B). By day 28 post-infection, BALB/c mice cleared the majority of parasites (Fig 3.1 A), in keeping with their intermediate/fast-responder phenotype (Lawrence and Pritchard 1994; Reynolds *et al* 2012). At this later time-point, $MIF^{-/-}$ mice harboured significantly higher parasite numbers than BALB/c (Fig 3.1 A), and this was also reflected in the faecal egg counts (Fig 3.1 B) which showed no diminution from day 14 levels in the same mice. This indicates that there is an ineffective immune response towards *H. polygyrus* in $MIF^{-/-}$ mice, from an early time point in infection.

The formation of granulomas around *H. polygyrus* larvae invading the submucosa of the small intestine has been shown to correlate strongly with strain related resistance to infection (see Chapter 2 & (Anthony *et al* 2007)). Thus, BALB/c had high numbers of granulomas, commonly 100-150, along the small intestine at day 14 post-infection, which declined in number to around 50 by day 28 (Fig 3.1 C). Unexpectedly, $MIF^{-/-}$ mice had the same number of granulomas as wild-type mice at both time points (Fig 3.1 C). This points to a function for granulomas other than direct larval killing, and demonstrates that MIF has no role in their formation.

3.2 Immune responses to *H. polygyrus* are comparable between $MIF^{-/-}$ and BALB/c mice

To examine whether the failure of immunity in the $MIF^{-/-}$ mice reflects an impairment of Th2 responsiveness to *H. polygyrus*, cytokine production by MLNC from infected mice was assessed. Levels of IL-4 (Fig 3.2 A) and IL-13 (Fig 3.2 B) released from MLNC restimulated with HES and αCD3, were comparable between BALB/c and

MIF$^{-/-}$ mice at day 14 post-infection. There was no defect in the ability to make these cytokines in MIF$^{-/-}$ mice, indeed they made significantly more IL-13 than BALB/c at day 14 post-infection with *H. polygyrus* (Fig 3.2 B). The inability to clear adult worms by day 28 is also not a reflection of an earlier skew towards a stronger Th1 response than BALB/c, as they made comparable levels of IFN-γ at day 14 (Fig 3.2 C).

Mast cells have been suggested to be a key cell type in the initiation and regulation of early Th2 responses to *H. polygyrus* in the gut, with mast cell deficient mice displaying higher susceptibility to the parasite (Hepworth *et al* 2012). Sections of small intestine from day 14-infected BALB/c and MIF$^{-/-}$ mice were stained with Toluidine blue, for clear metachromatic staining of mast cell granules. Mast cells were dyed deep-purple and were counted per villus for BALB/c and MIF$^{-/-}$ mice, with villus length being measured, to give a mast cell count/mm of villus (Fig 3.2 D). At day 14, there was no significant difference between the number of mast cells per mm of villus between BALB/c and MIF$^{-/-}$ mice, suggesting that MIF has no role in the distribution of mast cells in the gut during *H. polygyrus* infection and that the presence of mast cells does not necessarily protect against the parasite, at least in a MIF-deficient environment.

Regulatory T cell responses were also comparable between MIF$^{-/-}$ and BALB/c mice at days 14 and 28, in terms of the proportion of MLN cells with a CD4^{+} Foxp3^{+} phenotype (Fig 3.2 E), and the total number of cells expressing these markers (Fig 3.2 F). Proportions of T regs were significantly higher than naive levels at day 28 post-infection, and numbers of these cells increased throughout infection to 10x10^{6} at day 28. This corresponds with the similar Th1 and 2 responses observed in both groups upon infection, so that the susceptibility of MIF$^{-/-}$ cannot be explained by a Th subset deviation or expanded levels of regulatory cells.

As antibodies are known to be important in immunity to *H. polygyrus* (McCoy *et al* 2008; Wojciechowski *et al* 2009), titres of HES-specific IgM (Fig 3.2G C), IgG1 (Fig 3.2 H), and IgA (Fig 3.2 I) antibodies were measured and found to be

comparable between MIF$^{-/-}$ and BALB/c mice at day 28 post-infection, indicating that MIF deficiency does not result in any class-switching or antibody production defects after infection with *H. polygyrus*.

3.3 MIF is expressed in the granuloma, and intestinal epithelial cells, in BALB/c mice, following *H. polygyrus* infection

MIF is made by many different cell types including immune cells such as macrophages, T and B lymphocytes, DCs, eosinophils, mast cells, neutrophils and basophils, and by the epithelial cells lining the lung and gastrointestinal tract (Maaser *et al* 2002; Calandra and Roger 2003). It has been shown to have strong pro-inflammatory effects, and more specifically, to have important roles in promoting chemotaxis of immune cells to inflammatory sites (Wang *et al* 2006; Amano *et al* 2007; Santos *et al* 2011), and also enhancing wound healing (Hardman *et al* 2005). To localize expression of MIF in host tissues during infection, a polyclonal rabbit anti-serum to MIF was used in immunohistochemical staining of gut sections. This revealed MIF protein is present in the granulomas starting to form at day 6 post-*H.polygyrus* infection, and in epithelial cells of the small intestine of BALB/c mice (Fig 3.3). Predictably, this staining was not apparent in MIF-deficient mice, or in sections treated with an IgG control antibody. Staining was less intense in the fully-formed granuloma at day 14, suggesting a role for MIF in the initial recruitment or activation of cells in the intestine upon invasion of larvae into the mucosa. As the number of granulomas was no different between BALB/c and MIF$^{-/-}$ mice (Fig 3.1 C), MIF could act to recruit or activate certain types of cell in the granuloma of BALB/c mice, which may subsequently act to damage or kill the larvae. With such cell types absent or inactive in MIF-deficient mice, larvae may be able to escape unharmed into the lumen, establishing a long-term residence as adult worms.

3.4 Induction of macrophage activation markers is not affected in MIF-deficient mice

MIF has previously been shown both to be produced by macrophages (Calandra *et al* 1994), and to activate these cells. Thus, MIF sustains classical macrophage activation in the presence of Th1 skewing stimuli, such as endotoxin (Mitchell *et al*

2002) and protozoan parasitic infections (de Dios Rosado and Rodríguez-Sosa 2011; Bozza *et al* 2012). It is also involved in the alternative activation of macrophages in a Th2 setting (Prieto-Lafuente *et al* 2009), and in the tumour environment (Yaddanapudi *et al* 2013).

AAMΦ are a prominent cell type in the secondary *H. polygyrus* granuloma (Patel *et al* 2009), and are important intestinal effector cells in parasite clearance during reinfection (Anthony *et al* 2006). Less attention has been paid to the importance of AAMΦ in primary infection with *H. polygyrus*, although their accumulation in the primary granuloma has been shown to be less marked than in the granuloma forming after a secondary infection, in BALB/c mice (Anthony *et al* 2006).

To compare macrophage responses, two widely used markers of AAMΦ, RELM-α and Ym-1, were assayed over the first two weeks of infection. Both were found to be produced in the gut of BALB/c and $MIF^{-/-}$ mice, although at some early time points levels in $MIF^{-/-}$ animals appeared slightly lower (Fig 3.4 A, B), as confirmed in a subsequent experiment (see Fig 3.14). Over the full time course, quantitative PCR of cDNA from gut tissue did not identify significant differences between levels of transcript for RELM-α (Fig 3.4 C), or the enzyme Arg1 (Fig 3.4 D), both of which are highly expressed by AAMΦ (Loke *et al* 2002).

RELM-β has been shown to be an important effector molecule in innate protection against *H. polygyrus* and is released by intestinal epithelial cells to act directly to inhibit adult parasite feeding mechanisms (Herbert *et al* 2009). However, levels of RELM-β transcript were also found to be no different between BALB/c and $MIF^{-/-}$ mice (Fig 3.4 E).

cDNA levels of the above 3 characteristic innate type-2 molecules (RELM-α, Arg-1 and RELM-β) were for the most part upregulated upon infection and increased to peak at days 10-12 post-infection in both BALB/c and $MIF^{-/-}$ mice (Figs 3.4 C-E). MIF transcript, however, was relatively constant in BALB/c mice throughout infection and did not increase significantly above levels seen in naive mice, although a small peak was noted at day 8 post infection (Fig 3.4 F). This may be a reflection

of how MIF is made constiutively and stored in intracellular pools for rapid release upon stimulation (Calandra and Roger 2003).

3.5 Early innate immune responses to *H. polygyrus* are compromised in the MLN of MIF$^{-/-}$ mice

Total cell numbers in the MLN after infection with *H. polygyrus* revealed a slight delay in recruitment, or cell division, in MIF$^{-/-}$ mice compared to wild-type (Fig 3.5 A). The size of MLN, in terms of total cell number, increased steadily upon infection in both sets of mice, with comparable sizes of around 100x10^6 cells by day 14 and 28 post-infection. However, at the day 7 time-point, the number of MLN cells was significantly less in MIF-deficient mice compared to BALB/c, which may reflect the early effects of MIF as a chemoattractant to immune cells (Bernhagen *et al* 2007).

MIF has previously been linked to the activation and recruitment of several innate cell types in type-2 inflammatory situations, including macrophages (Falcone *et al* 2001; Yaddanapudi *et al* 2013) and eosinophils (Magalhães *et al* 2009; Yoshihisa *et al* 2010; Das *et al* 2011). Recently, MIF has also been linked to MDSCs (Simpson *et al* 2012), a heterogeneous set of innate cells, often investigated in tumour settings in mice and humans, for their ability to suppress T cell responses (Youn *et al* 2008; Duffy *et al* 2013). MIF is found to be highly concentrated in tumours, and is thought to contribute to cell migration and growth of tumours through its inflammatory chemokine activity (Bach *et al* 2008; Winner *et al* 2008; Shin *et al* 2012). MIF was found to induce highly suppressive monocytic MDSCs in the tumour microenvironment, which promoted tumour growth and metastasis (Simpson *et al* 2012). Furthermore, granulocytic MDSCs have recently been implicated in gastrointestinal helminth infection, as a key cell type mediating clearance of *N. brasiliensis* (Saleem *et al* 2012). Therefore, both subsets of MDSCs were further analysed in the MIF$^{-/-}$ infection model (Fig 3.5 B).

Being precursors of mature myeloid cells, MDSCs generally express CD11b and Gr1, but have been further divided into CD11b$^+$ cells that express either Ly6C (also termed monocytic MDSCs) or Ly6G (granulocytic MDSCs) (Youn *et al* 2008;

Peranzoni *et al* 2010). In $MIF^{-/-}$ mice, there was no difference in the proportion of $CD11b^{+}$ cells in the MLN when compared to BALB/c mice at day 7 post-infection with *H. polygyrus* (data not shown). However, within the $CD11b^{+}$ population, both $Ly6C^{+}$ $Ly6G^{-}$ (Fig 3.5 C) and $Ly6G^{+}$ $Ly6C^{-}$ (Fig 3.5 D) subsets were induced in the MLN at this time-point, and in both cases, represented around 0.3% of the total MLN cells. These cells were not induced in $MIF^{-/-}$ mice, with proportions not rising above naïve levels. A population that is double positive for both Ly6C and Ly6G is identified in the MLN of both naïve and infected mice (Fig 3.5 B), although infection does not increase the proportions of cells with this phenotype, and the difference between BALB/c and $MIF^{-/-}$ mice in an infection setting is less profound than the single positive populations (Fig 3.5 E). The total numbers of all 3 Ly6 subsets of $CD11b^{+}$ cells are significantly lower in $MIF^{-/-}$ mice than BALB/c mice when infected with *H. polygyrus* (Fig 3.5 F-H). Although further phenotyping by FACS can be undertaken to identify these cells, it is difficult to distinguish $Ly6C^{+}$ MDSCs from inflammatory monocytes, and $Ly6G^{+}$ MDSCs from neutrophils (Hickey 2012; Rose *et al* 2012; Lee *et al* 2013; Pillay *et al* 2013), without undertaking T cell suppression assays *in vitro*.

Another set of cells only recently described, but seen as key to the initiation of immune responses, are ILCs (Saenz *et al* 2010a; Spits *et al* 2013). Several subsets have been named, and group 2 ILCs (ILC2s) are most relevant in terms of helminth parasite settings, as they secrete IL-5 and IL-13 in response to IL-25, IL-33 and TSLP, thereby promoting the necessary Th2 responses needed for parasite clearance (Fallon 2006; Humphreys *et al* 2008; Moro *et al* 2010; Neill *et al* 2010; Saenz *et al* 2010b). These cells are typically identified by staining negatively for a panel of lineage markers, designating them as non-T and B cells, and non-myeloid cells. Intracellular IL-5 and IL-13 are measured alongside staining for cell surface markers, including ICOS, Sca1 and IL-33 receptor T1/ST2 (Spits *et al* 2013).
ILCs represent a very small proportion of total MLN cells, but nevertheless, were induced in the BALB/c upon infection with *H. polygyrus* and were absent in $MIF^{-/-}$ mice at day 7 post-infection, as also represented in total numbers of ILC2s in the MLN (Fig 3.5 I-K). A deficiency in ILC2s could explain the susceptibility of $MIF^{-/-}$

mice to *H. polygyrus*, although the robust Th2 response made by these mice suggests a more subtle effect than purely T cell cytokine release later on in infection.

3.6 Early innate immune responses to *H. polygyrus* are compromised in the PL of MIF$^{-/-}$ mice

As well as the intestinal draining lymph nodes and other lymphoid tissues, Th2 cells and associated IL-4 producing granulocytes, such as eosinophils and basophils, disseminate systemically to non-lymphoid regions of the body following *H. polygyrus* infection (Mohrs *et al* 2005). Using IL-4 reporter mice (Mohrs *et al* 2001), it has been demonstrated that, even in this non-migratory helminth model, that is entirely restricted to the intestine during its lifecycle, innate Th2-associated cells are responsible for the majority of IL-4 production in the peritoneal cavity, liver and pleural cavity (Mohrs *et al* 2005).

PL was therefore taken from naïve and d7 *H. polygyrus*-infected mice, and total numbers of cells found to be significantly less in MIF$^{-/-}$ mice compared to wild-type (Fig 3.6 A). BALB/c PL cell numbers increased after infection to around $20x10^6$ whereas those in MIF-deficient mice did not rise significantly above naïve levels. This may again reflect the function of MIF as a chemokine for inflammatory immune cells, as in the MLN above.

Eosinophils have been widely documented to be both producers of MIF (Rossi *et al* 1998) and to respond strongly to its characteristic chemoattractant properties (Magalhães *et al* 2009; Yoshihisa *et al* 2010). Indeed, eosinophils have been found to be largely absent in MIF-deficient mice in a number of models including *Schistosoma* liver and intestinal granulomas (Magalhães *et al* 2009), allergen-induced skin inflammation (Yoshihisa *et al* 2010) and allergic airway inflammation (Wang *et al* 2006; Magalhães *et al* 2007). The proportion of PL cells found to be eosinophils (SiglecF$^+$ CD11b$^+$) was significantly lower in MIF$^{-/-}$ mice compared to BALB/c (Fig 3.6 B), reflecting the role of MIF as an eosinophil chemoattractant. This was also reflected in the total numbers of eosinophils in the PL (Fig 3.6 C).

Analysis of $CD11b^+$ $Ly6C^+$ and $Ly6G^+$ populations revealed that increases in proportions of these cell types was not as profound in the PL at day 7 post-infection compared to in the MLN (Fig 3.6 D-F). The doubling in proportion of $Ly6G^+$ $Ly6C^-$ $CD11b^+$ cells in the PL of BALB/c mice was the only increase to reach statistical significance (Fig 3.6 E). However, when represented as total cell numbers, both single positive Ly6 populations (Fig 3.6 G, H) and the double positive population (Fig 3.6 I) were significantly lower in $MIF^{-/-}$ mice, reflecting the general lack of cell recruitment to this site upon *H. polygyrus* infection in these mice (Fig 3.6 A). This observation is also true for IL-5^+ ILCs, in that the proportion of cells with this phenotype in the PL is very low across all groups of mice assessed (Fig 3.6 J), although absolute numbers of ILC2s is significantly lower in $MIF^{-/-}$ mice (Fig 3.6 K).

3.7 The memory response to *H. polygyrus* is directed against adult egg production, but does not result in adult parasite clearance in $MIF^{-/-}$ mice

The secondary response to *H. polygyrus* results in a rapid and effective clearance of adult parasites in immunocompetent mice, which is reliant on both antibodies (McCoy *et al* 2008), and macrophages (Anthony *et al* 2006). In the case of antibody, different roles in controlling the parasite have been found, with parasite-specific antibodies acting to control tissue dwelling larval numbers, and polyclonal antibodies acting to reduce worm fecundity, to prevent the spread of parasites in the population (McCoy *et al* 2008).

As expected from previous publications, after clearance of an initial infection with pyrantel embonate dewormer, and secondary infection with *H. polygyrus,* BALB/c mice retained almost no worms at day 21 (Fig 3.7 A). In comparison, MIF deficiency rendered mice susceptible to higher parasite loads in both primary and secondary infections. As in primary infection, $MIF^{-/-}$ mice were not deficient in granuloma formation and had similar numbers to BALB/c mice at day 21-post secondary infection (Fig 3.7 B).

By day 21-post primary infection, parasite egg numbers in faecal material from BALB/c mice were very low and were reduced to zero in secondary infection (Fig

3.7 C). Although egg numbers were high in primary infection in MIF$^{-/-}$ mice, they are very significantly reduced in secondary infection, to levels barely above those in wild-type mice. This indicates that a MIF-independent immune response is able to substantially reduce worm fitness and egg output, but cannot complete clearance of adult parasites.

3.8 MIF deficiency renders mice more susceptible to *N. brasiliensis* infection, than BALB/c wild-type mice

To ascertain whether the compromised immunity to *H. polygyrus* in MIF$^{-/-}$ mice was extended to other parasite infections, the more acute gastrointestinal helminth model of *N. brasiliensis* was used. Two strains of *N. brasiliensis* were tested, having been passaged through either rats or mice in the maintenance of the lifecycle. The strain maintained in rats is more rapidly cleared from mice, so that by day 6 post-infection with 250 L3 larvae, BALB/c have cleared almost all worms from the small intestine (Fig 3.8 A). However, at this time point, MIF$^{-/-}$ mice harboured 20-60 adult worms (Fig 3.8 A). This is also illustrated by faecal egg counts, with MIF$^{-/-}$ mice having a significantly higher number of eggs than BALB/c (Fig 3.8 B).

The mouse-adapted strain of *N. brasiliensis* showed a stronger establishment in BALB/c mice, with numbers of adult worms remaining in the intestine at day 6 post-infection decreasing to zero by day 9 (Fig 3.8 C). MIF$^{-/-}$ mice had higher numbers of worms at all time points tested, and still harbored worms at day 12 post-infection (Fig 3.8 C). Egg numbers counted from BALB/c faecal material were consistently low, with those from MIF$^{-/-}$ mice significantly higher at the day 6 time point, and decreasing by day 9 (Fig 3.8 D).

The use of both strains of parasite illustrates that MIF-deficiency renders mice more susceptible to *N. brasiliensis* as well as *H. polygyrus*, despite the differences in migration and length of lifecycle within the mouse.

3.9 Cytokine responses are comparable between MIF$^{-/-}$ and BALB/c mice after *N. brasiliensis* infection

As with *H. polygyrus*, Th2 responses of day 7 *N. brasiliensis*-infected MIF$^{-/-}$ mice were comparable to those of BALB/c mice, as measured by IL-4 (Fig 3.9 A), IL-10 (Fig 3.9 B) and IL-13 (Fig 3.9 C) release from MLNC restimulated with NES and αCD3. There was also little difference in levels of IFN-γ (Fig 3.9 D).

As discussed above, AAMΦ have been found to be important, in *N. brasiliensis* infection, in intestinal smooth muscle hyper-contractility and resulting adult worm expulsion from the intestinal lumen (Zhao *et al* 2008). The presence of macrophage alternative activation markers RELM-α and Ym1 was measured in both lung and gut tissue homogenates, as *N. brasiliensis* migrates through the lung *en route* to the intestine. MIF$^{-/-}$ mice showed the same levels of both RELM-α (Fig 3.9 E, F) and Ym-1 (Fig 3.9 G, H) in both tissue sites, as BALB/c mice, indicating that the release of these proteins is not affected by MIF deficiency, and that their expression is not sufficient to cause worm expulsion.

3.10 Eosinophils and other innate populations are reduced in the MLN of MIF$^{-/-}$ mice, upon infection with *N. brasiliensis,* compared to wild-type mice

Eosinophils are an important cell type recruited to the lung (Voehringer *et al* 2004) and peritoneal cavity (Ohnmacht *et al* 2007) during *N. brasiliensis* infection. They are major producers of IL-4 and, have been show to have a key role in protection against this parasite (Shin *et al* 1997; Behm and Ovington 2000; Voehringer *et al* 2004; Voehringer *et al* 2006). BALB/c mice showed significant induction of eosinophils (SiglecF^{+} CD11b^{+}) in the MLN after 6 days of *N. brasiliensis* infection, compared to naïve levels (Fig 3.10 A). MIF$^{-/-}$ mice failed to induce eosinophilia in the MLN at this time point, and had significantly lower proportions and absolute numbers of eosinophils than BALB/c mice (Fig 3.10 A, B).

CD11b^{+} Ly6 cell phenotypes were analysed in the MLN at day 6 post-infection with *N. brasiliensis,* as these populations were greatly reduced early in *H. polygyrus* infection in the same site (Fig 3.5). Numbers of both CD11b^{+} Ly6C^{+} and CD11b^{+}

$Ly6G^+$ populations, and $Ly6C^+Ly6G^+$ double-positive cells were significantly increased upon infection in BALB/c mice, and this induction was greatly reduced in $MIF^{-/-}$ mice (Fig 3.10 C-E).

Proportions and absolute numbers of IL-5-producing ILCs were very low in the MLN at day 6 post-infection with *N. brasiliensis* (data not shown). As ILC2s are thought to be the key cell type in initiating Th2 responses, and *N. brasiliensis* is an acute model of helminth infection, this population was assessed at the earlier time-point of day 3 post-infection. There is a small increase in the proportion of $IL\text{-}5^+$ ILCs in BALB/c mice upon infection, although this was not statistically significant, and was not seen in $MIF^{-/-}$ mice (Fig 3.10 F). No difference was apparent between any group analysed when absolute numbers were enumerated (Fig 3.10 G).

3.11 Immune responses in the lung after *Alternaria* antigen administration are comparable between $MIF^{-/-}$ and BALB/c wild-type mice

The failure to induce ILC responses in $MIF^{-/-}$ mice in *H. polygyrus* could reflect an impaired ability to upregulate production of, and to respond normally to, the key ILC2-inducing cytokines IL-25 and IL33. To test this, the pathway was stimulated *in vivo* with the fungal allergen *Alternaria alternata* which has been found in mouse models of airway inflammation to be a potent inducer of IL-33 in the lung (Havaux *et al* 2005; Bartemes *et al* 2012; Mirchandani *et al* 2012; Walker *et al* 2013) and subsequent upregulation of IL-5 and IL-13-producing ILCs (Bartemes *et al* 2012; Salmond *et al* 2012). Utilising this model, IL-33 responses were measured in the lung of BALB/c and $MIF^{-/-}$ mice following intranasal administration of *Alternaria* (Fig 3.11 A). IL-33 was highly upregulated 1 hour after *Alternaria* exposure, in both the bronchioalveolar lavage fluid and in the lung tissue homogenate, but levels were no different between BALB/c and MIF-deficient animals.

Subsequently, at 48 hours after *Alternaria* exposure, cell populations in the lung were analysed, to assess effects of IL-33 release earlier in the response. The proportion of $IL\text{-}5^+$ ILCs in the lung cell digest from *Alternaria*-exposed mice did increase over levels in mice given PBS alone, in both BALB/c and MIF-deficient

mice (Fig 3.11 B). However, background levels in PBS exposed mice where high, and so differences were not found to be significant. In both BALB/c and MIF$^{-/-}$ mice levels rose from around 5% to 7% of lung cells having an IL-5^{+} ILC phenotype. IL-13^{+} ILCs were scarce and did not represent a recognizable population in the lung digest in these studies (data not shown).

Previous studies have shown that *Alternaria* induces RELM-α in the lung epithelium (Doherty *et al* 2012), and RELM-α^{+} AAMΦ are hallmarks of type-2 inflammation, including that in the lung (Nair *et al* 2009). As MIF has previously been implicated in the alternative activation of macrophages in a Th2 setting (Prieto-Lafuente *et al* 2009), the proportion of these cells was also assessed in lung digest 48 hours after *Alternaria* exposure. RELM-α^{+} alveolar macrophages (CD11b^{low}, CD11c^{high}, F4/80^{+}) were indeed induced upon *Alternaria* exposure in BALB/c mice, but induction was significantly reduced in MIF-deficient mice (Fig 3.11 C).

3.12 MIF$^{-/-}$ mice can respond to IL-25 and IL-33 to the same extent as BALB/c mice

Another mechanism by which MIF deficiency could result in reduced ILC induction could be the failure to respond to the key cytokines IL-25 and IL-33. IL-25 is known to induce ILC2s in the early stages of innate responses and intraperitoneal administration of the recombinant protein elicits ILCs in the MLN (Saenz *et al* 2010b). Using this protocol, small increases in the absolute numbers of IL-5^{+} (Fig 3.12 A) and IL-13^{+} (Fig 3.12 B) ILCs in the MLN were observed, in both BALB/c and MIF$^{-/-}$ mice, suggesting that MIF-deficiency does not lead to a failure in ILC2 induction in response to IL-25.

Recombinant IL-33 was administered intranasally, in a protocol designed to directly induce ILCs in the lung (Bartemes *et al* 2012). IL-33-induced airway inflammation was seen in both BALB/c and MIF$^{-/-}$ mice to the same extent, when cells digested from 1 lobe of the lung were enumerated (Fig 3.12 C). IL-33 also induced a significant increase in the number of RELM-α^{+} alveolar macrophages in BALB/c and a similar increase, although not significant, in MIF$^{-/-}$ mice (Fig 3.12 D). Absolute

numbers of both IL-5^{+} and IL-13^{+} ILCs were found to increase after IL-33 administration, and there was no defect in MIF$^{-/-}$ mice – indeed they displayed a more significant increase from PBS control levels than BALB/c (Fig 3.12 E, F). Neither IL-25 nor IL-33 administration protocols revealed a defect in MIF$^{-/-}$ mice to induce ILCs in response to these cytokines. As the phenotype of impaired ILC generation in MIF-deficient mice is only observed in the context of *H. polygyrus* infection, it could be proposed that MIF is acting redundantly with another pathway that is inactivated by the parasite *in vivo.*

3.13 Intra-peritoneal administration of rMIF fails to reverse the MIF$^{-/-}$ phenotype in *H. polygyrus* infection

In an attempt to reverse the susceptible phenotype of MIF$^{-/-}$ mice, recombinant MIF was administered i.p., every 2 days for the first week of *H. polygyrus* infection. A BALB/c control group was used as a resistant baseline, and parasitological outcomes were measured. At day 28 post-infection, as expected BALB/c mice had few adult parasites in the small intestine, and MIF$^{-/-}$ mice given PBS as a control were susceptible, with significantly more worms than BALB/c mice (Fig 3.13 A). Surprisingly, rMIF administration led to a higher worm burden in MIF$^{-/-}$ mice. Faecal egg counts were variable in the PBS-treated group but both MIF$^{-/-}$ groups had higher egg counts than the BALB/c controls (Fig 3.13 B). As has been previously observed, BALB/c and MIF$^{-/-}$ mice control groups had similar numbers of granulomas at day 28, but unexpectedly, rMIF administration resulted in decreased granuloma numbers in MIF$^{-/-}$ mice (Fig 3.13 C).

The above observations were not mirrored when rMIF was administered i.p. at a later time point in infection, from day 14. Worm (Fig 3.13 D), egg (Fig 3.13 E) and granuloma counts (Fig 3.13 F) were comparable between PBS and rMIF-treated MIF$^{-/-}$ mice in this case, being more susceptible than BALB/c and having similar numbers of intestinal granulomas.

These results suggest a role for MIF in the first week of infection, rather than later, but the unexpected increase in susceptibility, may result from its administration in a

non-physiological location, too distant from the normal site at which MIF is acts in a normal infection. For example, placing rMIF in the peritoneal cavity may provide a chemoattractant signal which draws away cells which are normally involved in the formation of granulomas on the intestinal wall, thereby decreasing the frequency of them in $MIF^{-/-}$ mice treated with MIF. This would in turn result in less larval damage in the intestinal mucosa by cells in the granulomas, and greater adult worm survival at day 28.

3.14 Intra-peritoneal administration of rMIF induces the alternative activation of macrophages in $MIF^{-/-}$ mice, after *H. polygyrus* infection

In view of the unexpected observations in parasite survival and granuloma formation in the above experiments, the same protocol of rMIF administration was conducted, but with immunological parameters assessed at day 7 post-infection. RELM-α and Ym1 protein levels in both gut homogenate and PL were measured by ELISA, and as expected, all measurements showed increases upon infection in BALB/c mice (Fig 3.14 A-D). Furthermore, upon administration of rMIF in BALB/c, although not found to be significant, there are clear increases in RELM-α and Ym1 in both gut and PL.

Control groups of $MIF^{-/-}$ mice given PBS had very low levels of RELM-α and Ym1 after *H. polygyrus* infection, but rMIF administration boosted production of these proteins, especially in gut tissue, to levels comparable to BALB/c mice (Fig 3.14 A, B). rMIF also increased the proportion of AAMΦ ($Ym1^{+}$ $F4/80^{+}$ $CD11b^{+}$) in the PL in $MIF^{-/-}$ mice, as assessed by FACS (Fig 3.14 E).

3.15 rMIF boosts proportions of ILCs in BALB/c mice and CD11b+ cell populations in $MIF^{-/-}$ mice

Proportions of ILCs in the PL and MLN were measured after 7 days of *H. polygyrus* infection and rMIF or PBS administration. As expected, levels of $IL\text{-}5^{+}$ and $IL\text{-}13^{+}$ ILCs increased after infection in BALB/c mice in both PL and MLN (Fig 3.15 A-D). Proportions were further boosted, although not significantly, by administration of rMIF. Contrary to the findings for AAMΦ, rMIF did not boost proportions of ILCs

in either PL or MLN in MIF-deficient mice (Fig 3.15 A-D), above levels seen in PBS control groups. These findings suggest that rMIF alone is not sufficient to reconstitute the ILC responses towards *H. polygyrus*, and that the MIF may be required in concert with a cell type, factor or physiological site not reproduced by the i.p. protocol.

The boosting effect of rMIF in BALB/c was also apparent when $CD11b^+$ cell subsets were assayed after *H. polygyrus* infection. In the MLN, although naïve levels were variable, proportions of $Ly6C^+$ $CD11b^+$ cells were boosted in both BALB/c and $MIF^{-/-}$ mice (Fig 3.15 E). Likewise, in the PL, $Ly6C^+$ $CD11b^+$ cells that $MIF^{-/-}$ mice failed to induce upon infection compared to BALB/c mice, were boosted upon rMIF administration (Fig 3.15 F).

Induction of $Ly6G^+$ $CD11b^+$ cells was less dramatic in BALB/c mice after infection in both the MLN and PL (Fig 3.15 G, H), although administration of rMIF to MIF-/- mice boosted this cell population in the MLN (Fig 3.15 G).

3.16 Administration of the MIF chemical inhibitor 4IPP renders BALB/c mice more susceptible to *H. polygyrus*

Chemical inhibitors of MIF have been developed, and these have 3 main modes of action; those that compete with the substrate for the catalytic site of MIF (Al-Abed *et al* 2005), those that disrupt the trimeric structure of the MIF molecule (Ouertatani-Sakouhi *et al* 2010), and those that covalently alter MIF at the catalytic (Senter *et al* 2002) or other site (Bai *et al* 2012). 4-iodo-6-phenylpyrimidine (4-IPP) is one such small molecule inhibitor, which acts as a suicide substrate for MIF, irreversibly covalently altering the biologically active catalytic NH_2-terminal proline (Winner *et al* 2008). It was found to be 5-10 times more potent than ISO-1, a well-characterized MIF antagonist (Lubetsky *et al* 2002; Al-Abed *et al* 2005), at blocking MIF-dependent cell migration and dopachrome tautomerase activity (Winner *et al* 2008) and so was utilized to chemically inhibit the function of MIF in wild-type BALB/c mice in parasite infection.

4-IPP was administered to BALB/c mice, i.p. every 2 days, in the first week of *H. polygyrus* infection, following a modified protocol used in previously published studies (Gadjeva *et al* 2010; Yaddanapudi *et al* 2013). The control group for infection, as expected, cleared the majority of adult worms from the intestine by day 28 post-infection (Fig 3.16 A). DMSO had no significant effect on worm burden, but when 4-IPP was administered in DMSO, worm burden was increased significantly above that in mice that had received no treatment, giving a mean worm burden of 40 worms in mice treated with the MIF inhibitor (Fig 3.16 A). This result was mirrored in faecal egg counts from these mice which increased over 5-fold over untreated controls (Fig 3.16 B). 4-IPP administration had no effect on granuloma formation (Fig 3.16 C), and as in the normal infection setting BALB/c and $MIF^{-/-}$ mice did not differ in the ability to form granulomas (Fig 3.1 C), this is not unexpected.

Administration of the DMSO vehicle alone had small effects on both worm and egg counts in these *H. polygyrus* infection experiments, but when compared to 4-IPP in DMSO, the effect was minimal.

In a different experiment, using the same protocol of 4-IPP administration during *H. polygyrus* infection, PL cellular populations were analysed at the earlier time point of day 7 post-infection. The proportion and total number of macrophages in the PL were significantly decreased, after 4-IPP administration, from levels in infected control mice (Fig 3.16 D, E). However, the effect of the DMSO vehicle alone was also significantly different from untreated mice. When PL was analysed by ELISA for RELM-α and Ym-1 content, the effect of DMSO alone was less profound, whereas the mice that were treated with 4-IPP made very significantly lower levels of these proteins than control mice (Fig 3.16 F). This shows that although DMSO alone may be having a negative impact on macrophages, only by inhibiting MIF, do levels of the key alternatively activated macrophage markers decrease.

$MIF^{-/-}$ mice have been shown to have defects in eosinophilia upon *H. polygyrus* infection in the PL (see Fig 3.7). Administration of 4-IPP to BALB/c mice during

infection was found to replicate the phenotype of $MIF^{-/-}$ mice, by significantly decreasing both proportions (Fig 3.16 G) and total numbers of eosinophils (Fig 3.16 H) in the PL, with no major effect of DMSO alone. This suggests that the negative effects of DMSO on macrophages may be cell specific.
$MIF^{-/-}$ mice also display defects in induction of ILC2s and CD11b+ myeloid populations in the MLN during *H. polygyrus* infection (see Fig 3.6). Although MLNC were not analysed in this experiment, the number of $IL\text{-}5^{+}$ ILCs in the PL appears to decrease slightly in the 4-IPP-treated group compared to untreated controls (Fig 3.16 I). The proportions (Fig 3.16 J) and total numbers (Fig 3.16 K) of $Ly6G^{+}$ $CD11b^{+}$ cells decreased very significantly upon 4-IPP treatment from untreated control levels, an effect not seen in $MIF^{-/-}$ animals in the PL (see Fig 3.6).

3.17 Administration of the MIF chemical inhibitor 4IPP renders BALB/c mice more susceptible to *N. brasiliensis*

The same protocol of 4-IPP administration was then carried out in the setting of *N. brasiliensis* infection. Although adult worm counts were consistently low in all mice, 4-IPP treatment significantly affected the ability of BALB/c mice to clear adult worms from the intestine by day 6 post-infection (Fig 3.17 A). A small increase in egg counts from the 4-IPP treated group was also observed (Fig 3.17 B).
BALB/c mice given 4-IPP during *N. brasiliensis* infection mimicked the phenotype of $MIF^{-/-}$ mice in their lack of eosinophil recruitment to the peritoneal cavity (Fig 3.17 C) confirming that MIF is directly involved in activation of these cells.

$IL\text{-}5^{+}$ ILC proportions were also significantly decreased in 4-IPP-treated BALB/c mice from levels in infected, but untreated, controls (Fig 3.17 D). No change was observed in $CD11b^{+}$ $Ly6C^{+}$ cell numbers between treated and untreated mice (Fig 3.17 E), but the administration of 4-IPP significantly reduced the proportion of $CD11b^{+}$ $Ly6G^{+}$ cells compared to levels seen in untreated controls (Fig 3.17 F).
As observed in *H. polygyrus* infections, treatment with the vehicle DMSO alone had some effect on cell proportions, although not as profound as 4-IPP in DMSO. Although DMSO is widely used as a low-toxicity solvent to deliver chemicals to humans and animals, it known to have a wide array of physiological effects and is

involved in multiple pathways (Kelava *et al* 2011). However, the addition of MIF-inhibitor to DMSO has an additional significant effect on several cellular populations in the PL, during *H. polygyrus* and *N. brasiliensis* infections.

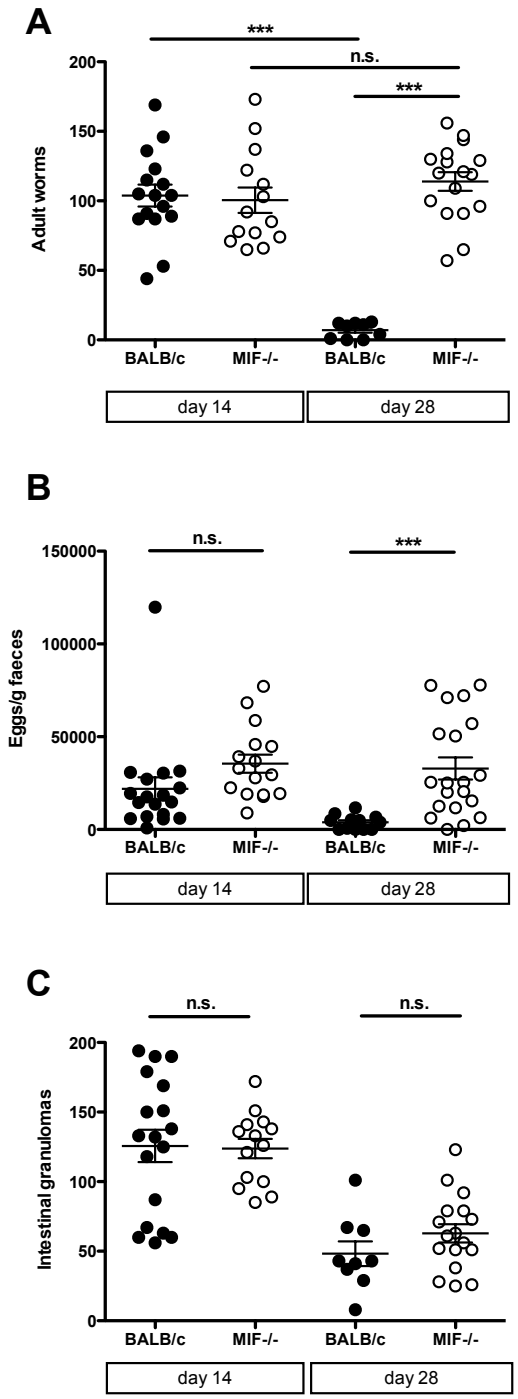


Fig 3.1 - MIF deficiency renders mice more susceptible to *H. polygyrus* than BALB/c WT mice

Female BALB/c and MIF$^{-/-}$ mice were infected with 200 *H. polygyrus* L3 larvae. Adult worm burden in the small intestine (A), eggs/g faecal material (B) and intestinal granulomas (C) were enumerated at days 14 and 28 post-infection.
Data shown are from 3 independent experiments combined. *** = $p<0.001$.

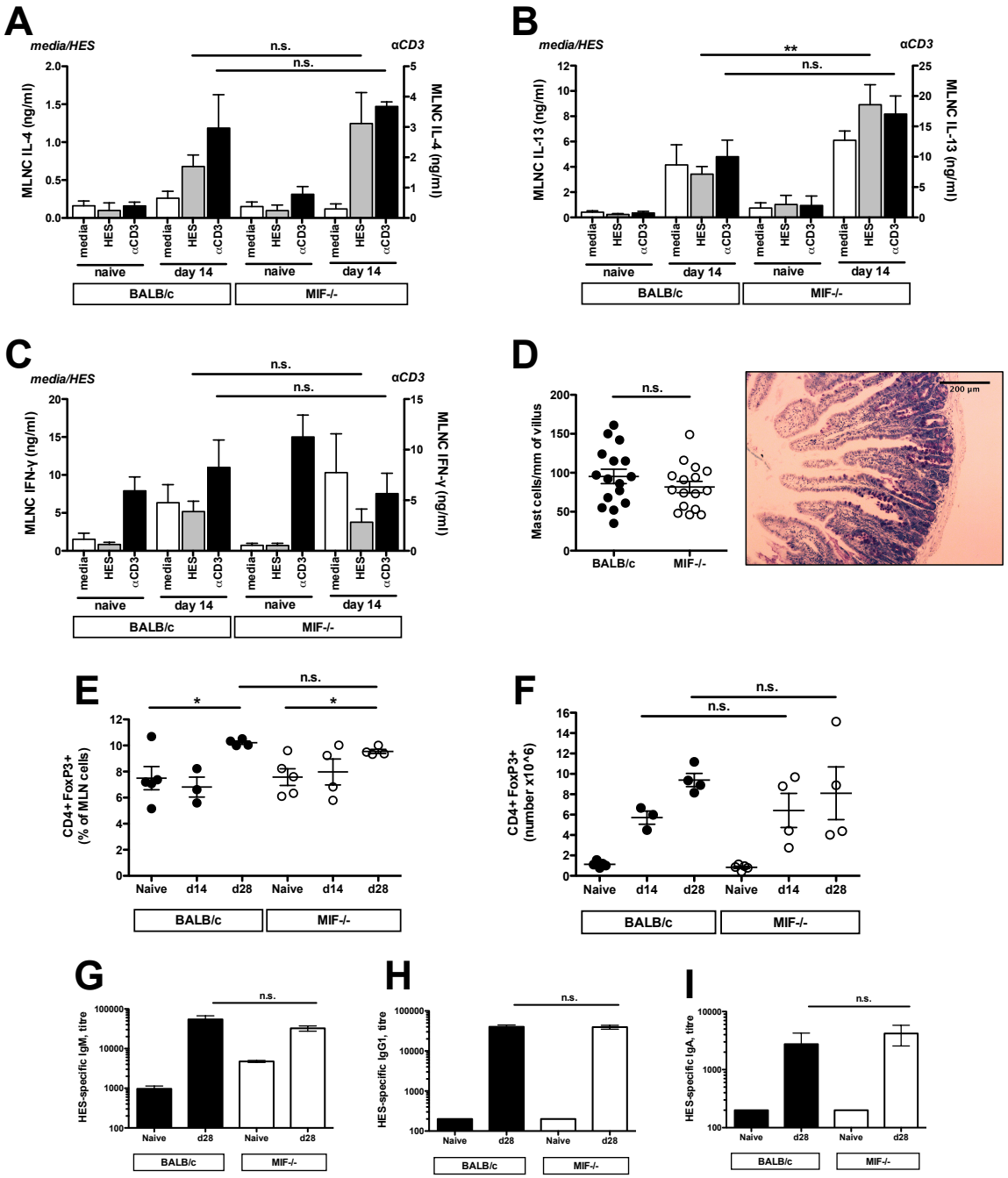


Fig 3.2 - Immune responses to *H. polygyrus* are comparable between MIF$^{-/-}$ and BALB/c mice

MLN from naive and d14 *H.polygyrus*-infected BALB/c and MIF$^{-/-}$ mice were restimulated with media, 1 µg/ml HES or 2 µg/ml αCD3 for 72 hours. Levels of IL-4 (A), IL-13 (B) and IFN-γ (C) were measured by ELISA. Results for media and HES restimulation are presented on the left Y axis, and those for αCD3 on the right. Results are combined from 2 independent experiments. Sections of small intestine from day 14-infected BALB/c and MIF$^{-/-}$ mice were stained with Toluidine blue to highlight mast cells. Scale bar on representative picture equals 200 µm. Mast cells were enumerated and are represented as cells per mm of villus measured (D). Each dot represents a complete villus section, with results collated from at least 3 different mice per group.
CD4^{+} FoxP3^{+} MLN cells were FACS stained from naive, d14 and d28-infected mice and are represented as proportions of total MLN cells (E) and total number (F). HES-specific serum IgM (G), IgG1 (H) and IgA (I) titres were measured by ELISA. Data are from one experiment. * = $p<0.05$, ** = $p<0.01$.

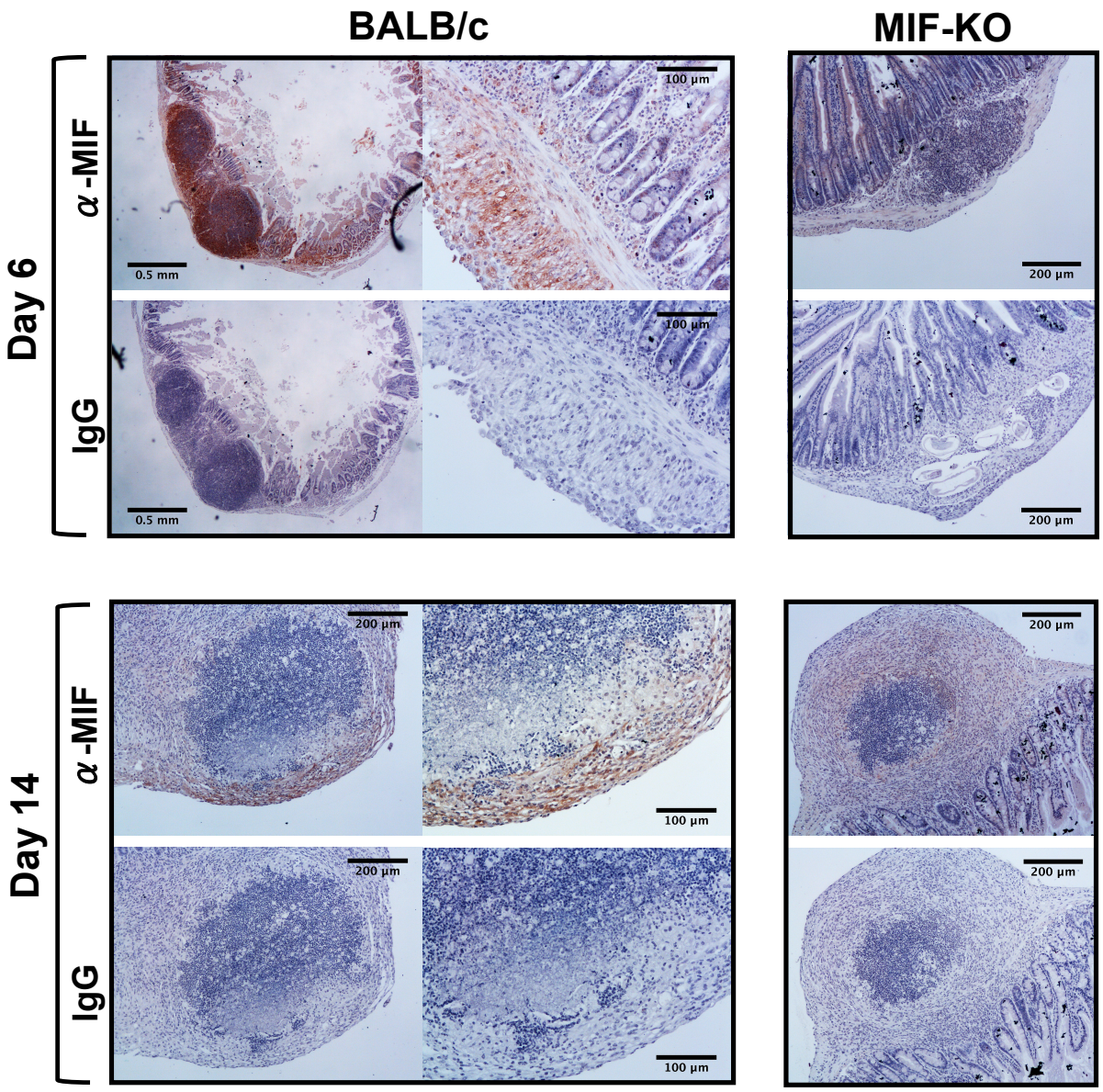


Fig 3.3 - MIF is expressed in the granuloma and intestinal epithelial cells, in BALB/c mice, following *H. polygyrus* infection

Small intestine from d6 and d14 *H.polygyrus*-infected BALB/c and MIF$^{-/-}$ mice were prepared as described for IHC staining. Polyclonal rabbit α-MIF was used to detect MIF on deparaffinised sections. IgG was used on control slides. Pictures were taken using a Leica compound microscope at either a x4, x10 or x20 objective; scale bars indicate 500, 200 or 100 µm respectively.

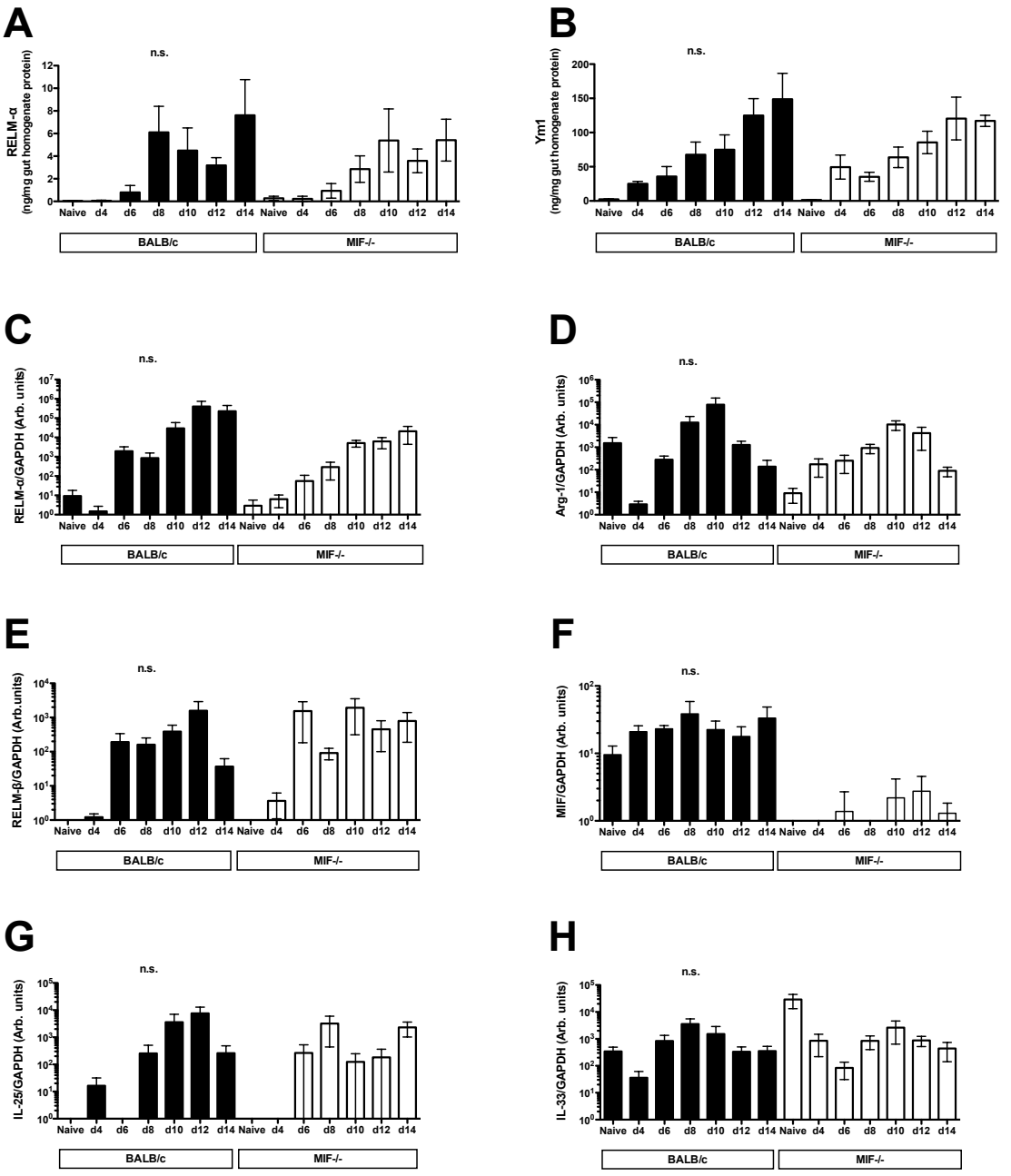


Fig 3.4 - Induction of early innate response markers is not affected in MIF-deficient mice

RELM-α (A) and Ym-1 (B) levels in gut homogenate from BALB/c and MIF$^{-/-}$ mice were analysed by ELISA, and normalised against total protein content. Results are combined from 2 independent eperiments.

cDNA made from RNA extracted from small intestine of BALB/c and MIF$^{-/-}$ mice was measured for RELM-α (C), Arg-1 (D), RELM-β (E), MIF (F), IL-25 (G) and IL-33 (H), and normalised against levels of the housekeeping gene GAPDH in each sample.

Results are representative of 2 independent experiments. Statistical tests compared BALB/c and MIF$^{-/-}$ levels for each time point, except for figure F which compared all time points to naive levels in BALB/c only. n.s. = no comparison on graph was significant ($p>0.05$).

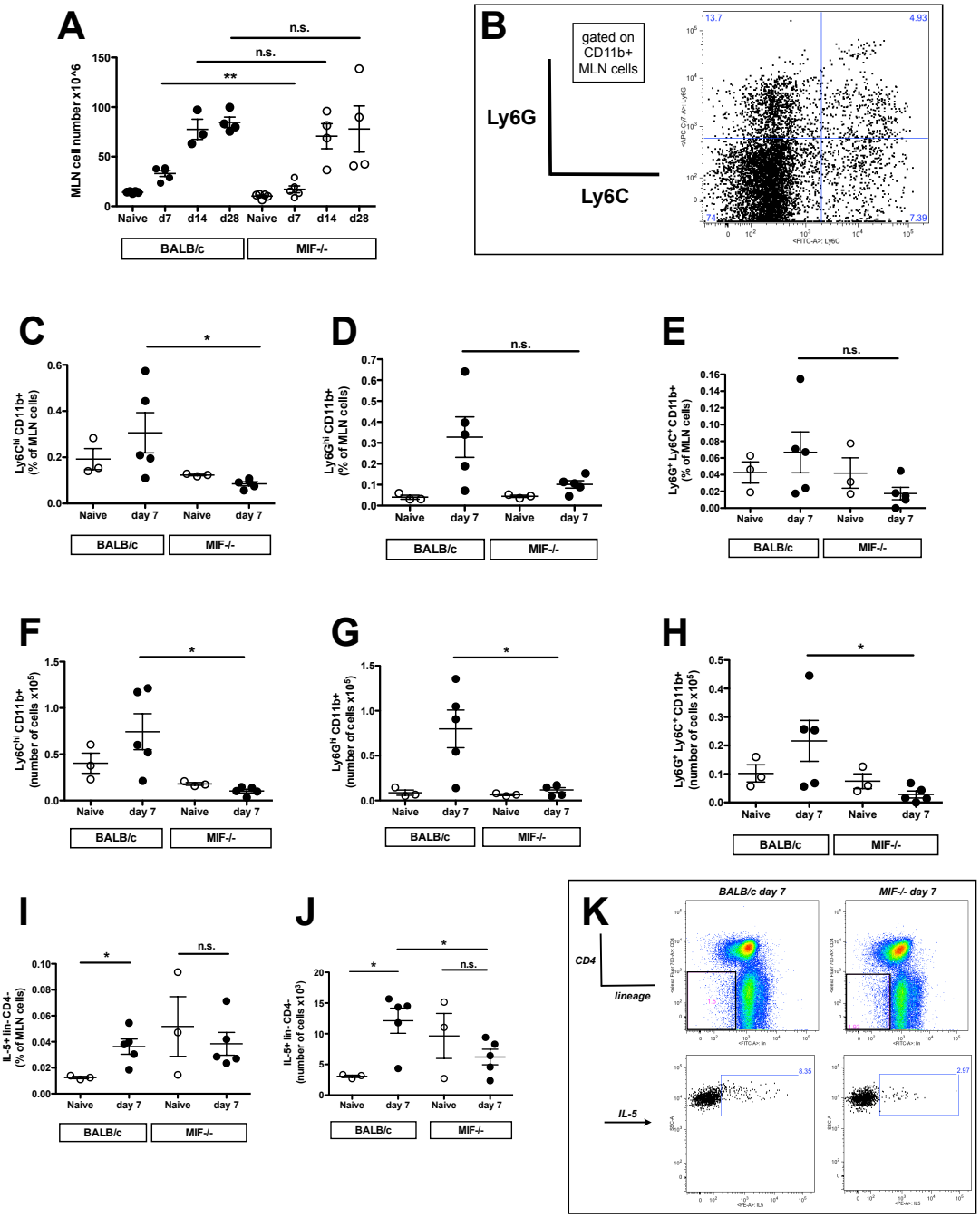


Fig 3.5 - Early innate immune responses to *H. polygyrus* are compromised in the MLN of MIF$^{-/-}$ mice

Total MLN cell numbers were enumerated from naive, d14 and d28 *H. polygyrus*-infected mice (A). Cells from naive and d7 only were stained for CD11b, Ly6C and Ly6G (B) and both single-positive and the double-positive population are representated as proportion of MLNC (C-E) and total numbers (F-H).
Cells were also stained for CD4, a lineage marker panel and intracellular IL-5 to assess ILC proportions (I) and total numbers (J). Representative FACS plots showing lineage negative gating and IL-5 staining within this population for d7-infected BALB/c and MIF$^{-/-}$ are represented (K).
Results are representative fo three independent experiments.* = p<0.05, ** = p<0.01.

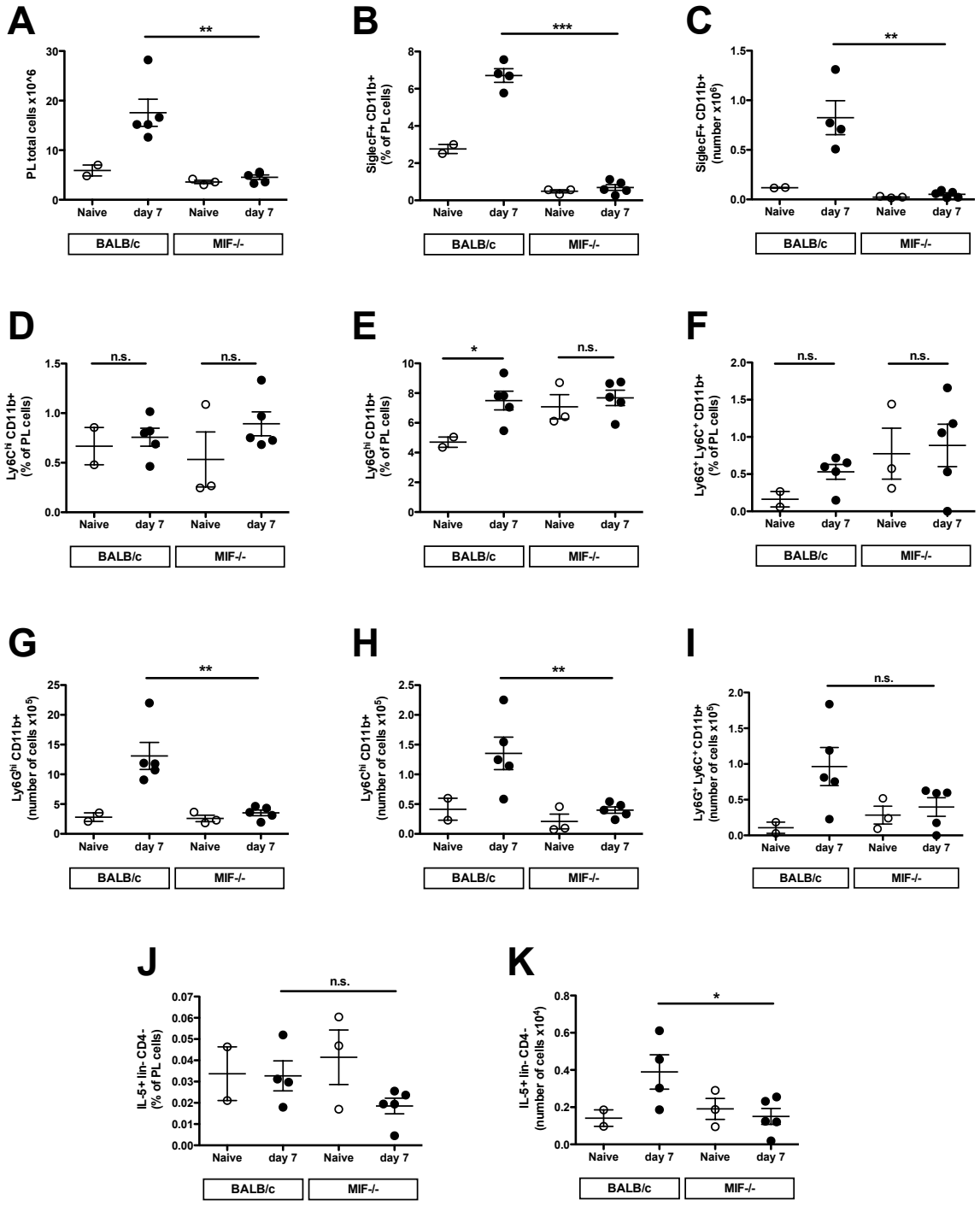
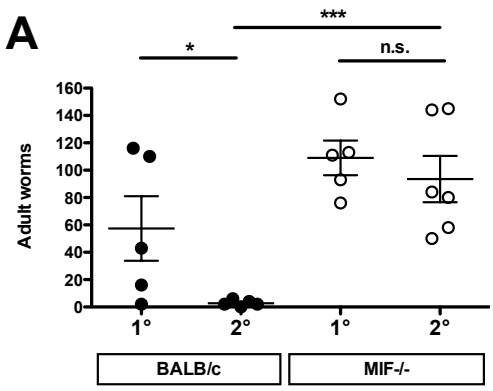
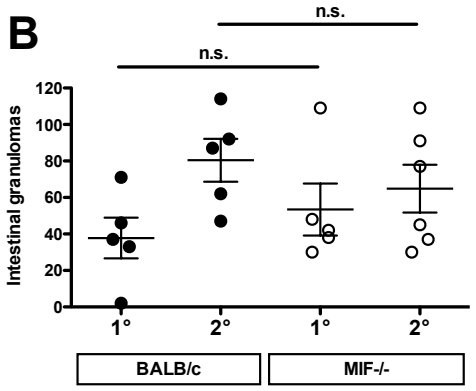


Fig 3.6 - Early innate immune responses to *H. polygyrus* are compromised in the PL of MIF$^{-/-}$ mice

Peritoneal lavage was taken from naive and d7 *H. polygyrus*-infected BALB/c and MIF$^{-/-}$ mice. Total cell numbers were enumerated (A), and the proportion and total numbers of eosinophils (CD11b and SiglecF) (B, C), proportions of CD11b^{+} Ly6C^{+} (D), Ly6G^{+} (E) and double positive cells (F), and total numbers of these populations (G-I) were assessed by FACS. Proportions and total numbers of IL-5^{+} ILCs (J, K) were also assessed.
Results are representative fo two independent experiments. * = p<0.05, ** = p<0.01, *** = p<0.001





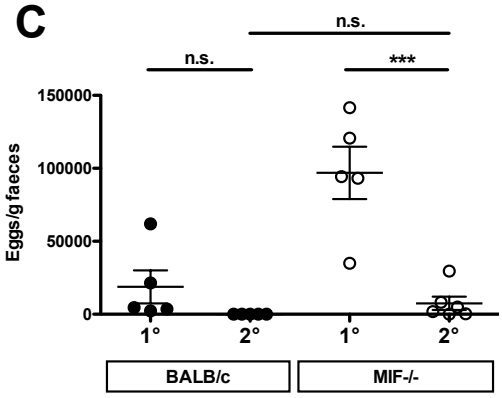


Fig 3.7 - The memory response towards *H. polygyrus* is directed against adult egg production, but does not result in adult parasite clearance in MIF$^{-/-}$ mice

Male BALB/c and MIF$^{-/-}$ mice were infected with 200 *H.polygyrus* L3 larvae. 28 days post-infection 2.5 mg of pyrantel embonate was administered in 200 µl water by oral gavage, twice over 24 hours. After 14 days, mice were reinfected (or infected for the first time in 1° groups). Adult worms (A), intestinal granulomas (B) and eggs/g faecal material (C) were enumerated at d21 post infection. Results are representative of 2 independent experiments * = $p<0.05$, *** = $p<0.001$

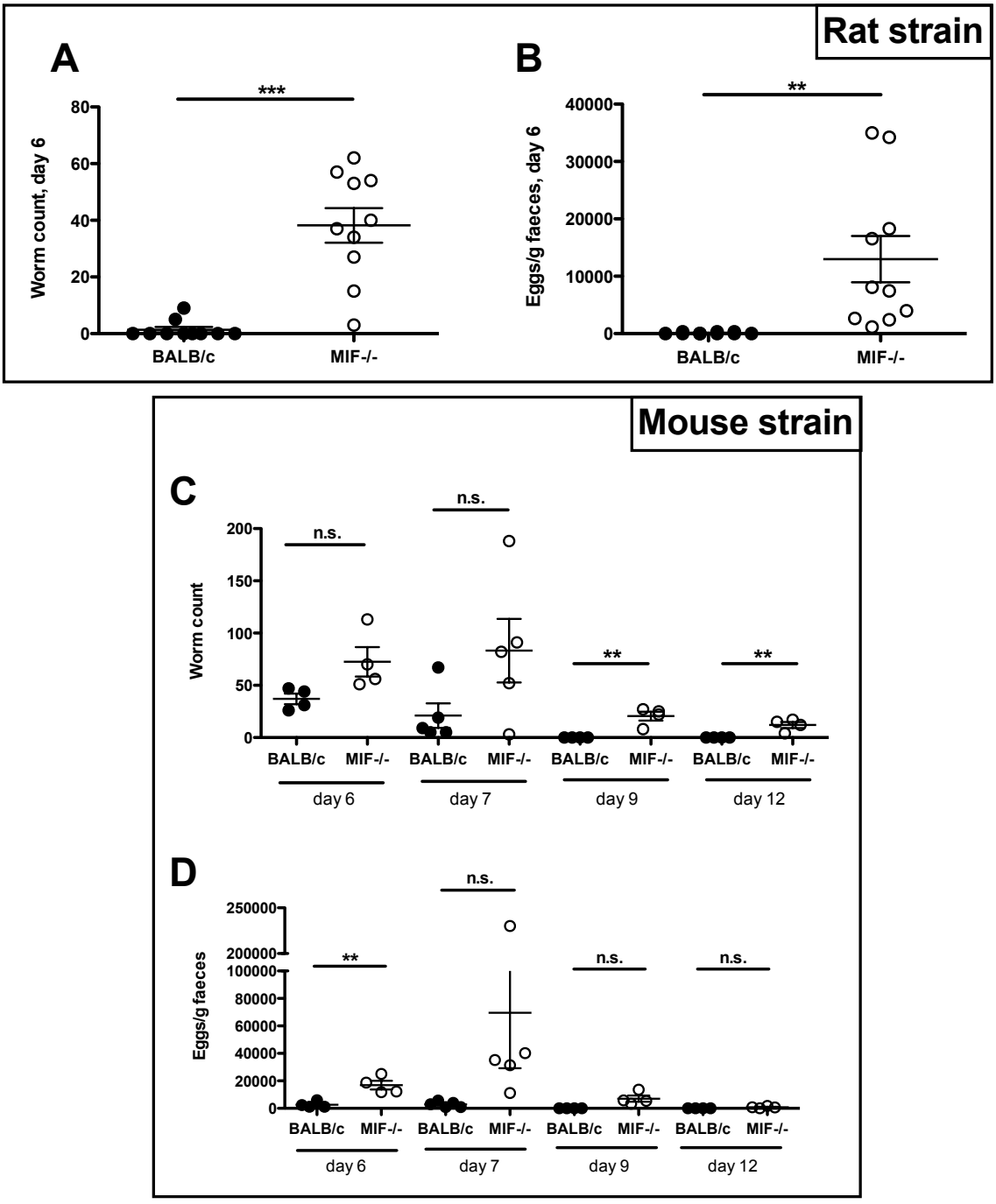


Fig 3.8 - MIF deficiency renders mice more susceptible to N. brasiliensis than BALB/c wild-type mice

Experiments were carried out using 250 *N. brasiliensis* L3 larvae, passaged through rats (A+B) or mice (C+D) during lifecycle maintenance. Adult worm burdens (A) and eggs/g faecal matter (B) were enumerated for the rat strain at day 6 post-infection - these data are combined from 2 independent experiments.
A time course was carried out using the mouse strain of *N. brasiliensis*, with worm and egg burdens enumerated at days 6, 7, 9 and 12 post-infection - data are from one experiment.
*** = $p<0.001$, ** = $p<0.01$.

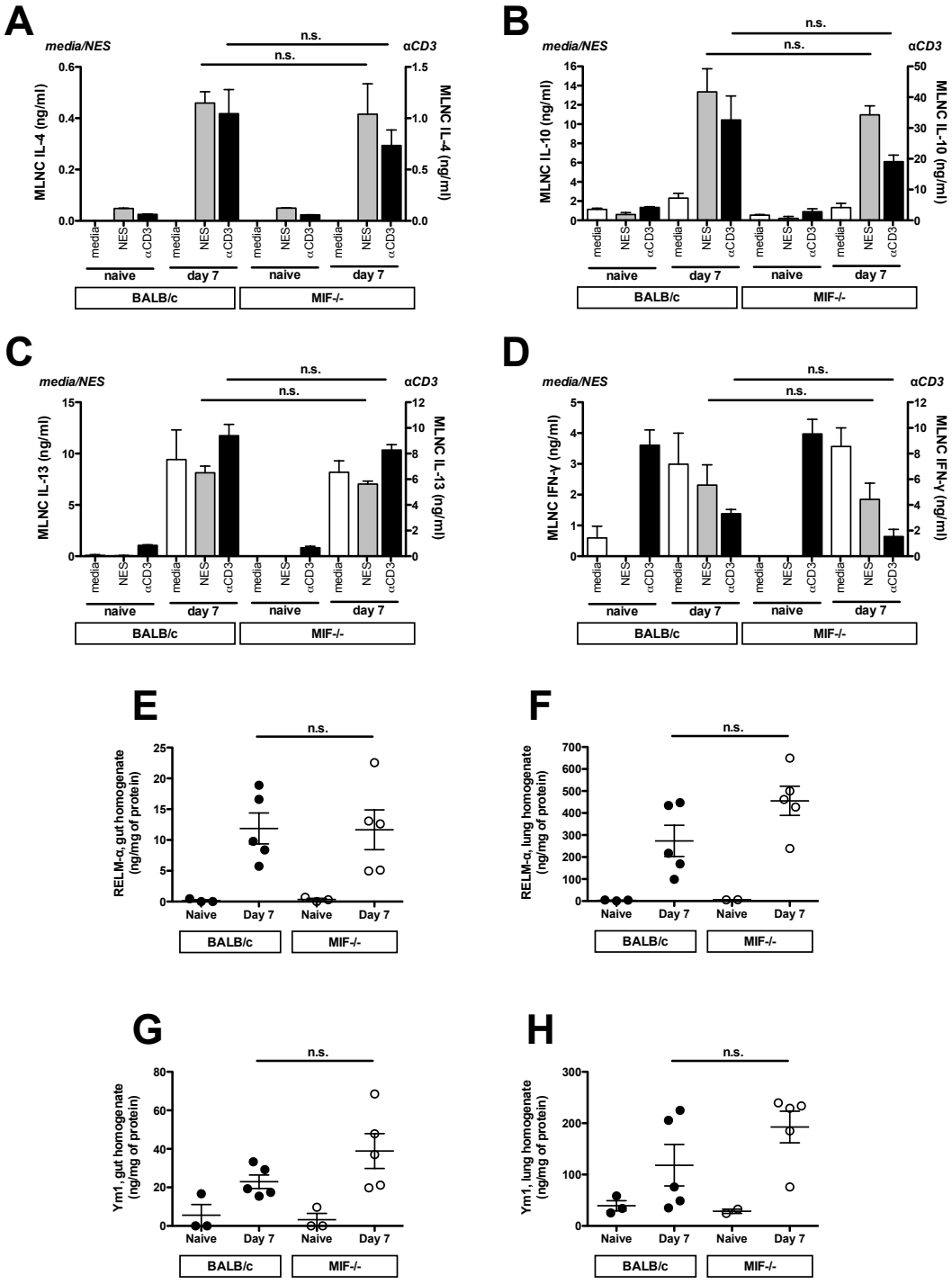


Fig 3.9 - Cytokine responses are comparable between MIF$^{-/-}$ and BALB/c after *N. brasiliensis* infection

MLN cells were harvested from naive and d7 *N. brasiliensis*-infected BALB/c and MIF$^{-/-}$ mice, and stimulated for 72 hours with NES, αCD3 or media alone (A-D). Supernatants were assessed by ELISA for IL-4 (A), IL-10 (B), IL-13 (C) and IFN-γ (D).
Small intestine and lung homogenate, prepared as described, were assessed by ELISA for the presence of RELM-α (E +F) and Ym1 (G+H) - data are presented as ng of cytokine per mg of protein in the sample (measured by Bradford assay).
The mouse strain of *N. brasiliensis* was used throughout. Data are representative of 2 independent experiments.

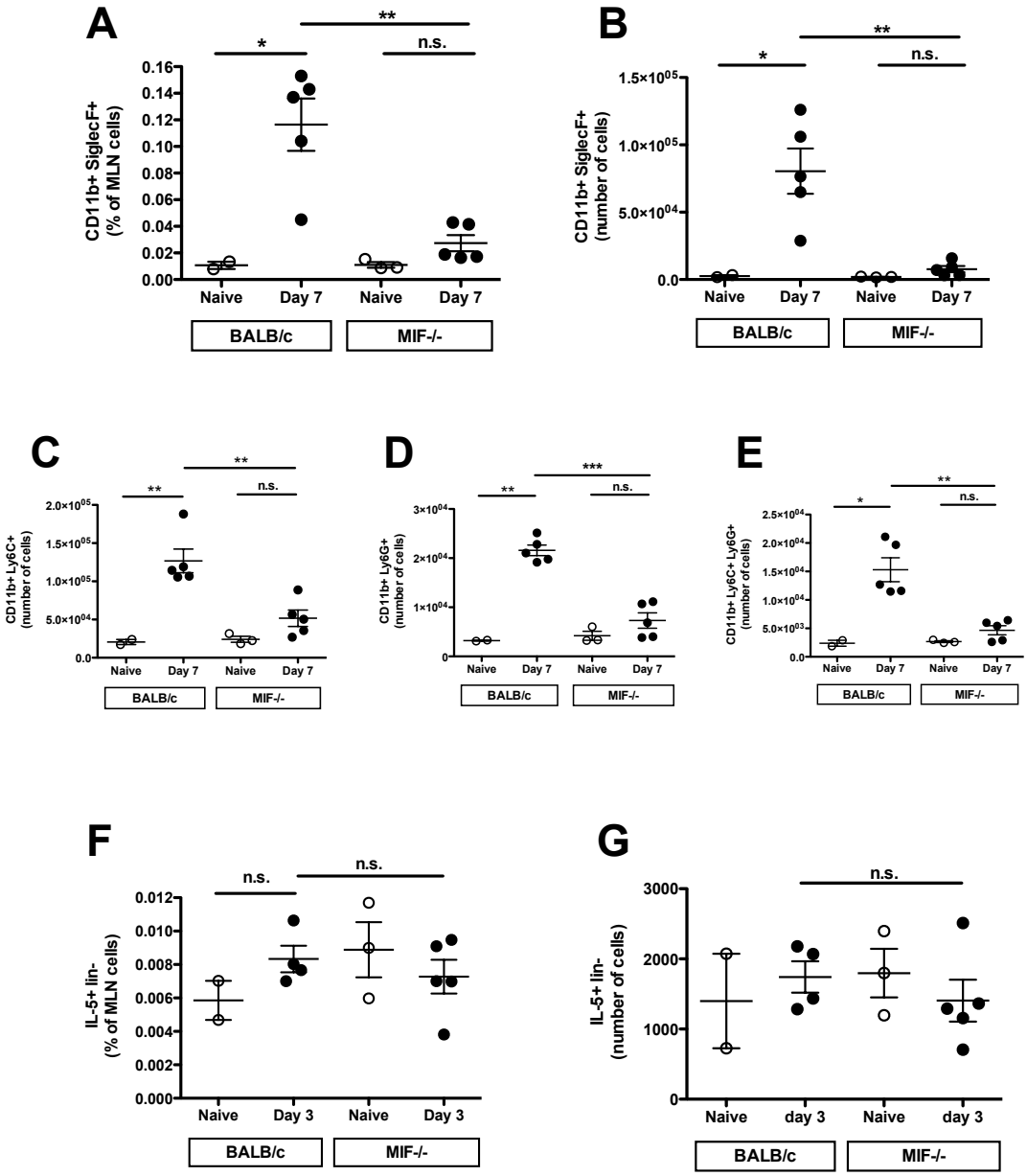


Fig 3.10 - Eosinophils and other innate populations are reduced in the MLN of MIF-/- mice, upon infection with *N. brasiliensis*, compared to wild-type mice

MLN cells were extracted from BALB/c and MIF$^{-/-}$ mice at day 7 post-infection with *N. brasiliensis,* and analysed by FACS to assess proportions and absolute numbers of eosinophils (CD11b$^+$ SiglecF$^+$)(A, B), absolute numbers of CD11b$^+$ Ly6C$^+$ cells (C), CD11b$^+$ Ly6G$^+$ cells (D) and CD11b$^+$ Ly6C/G double-positive cells (E). MLN cells from day 3 post-infection were assessed for proportions and absolute numbers of IL-5$^+$ ILCs (F, G).
The mouse strain of *N. brasiliensis* was used throughout. Results are representative of 2 independent experiments. * = p<0.05, ** = p<0.01, *** = p<0.001

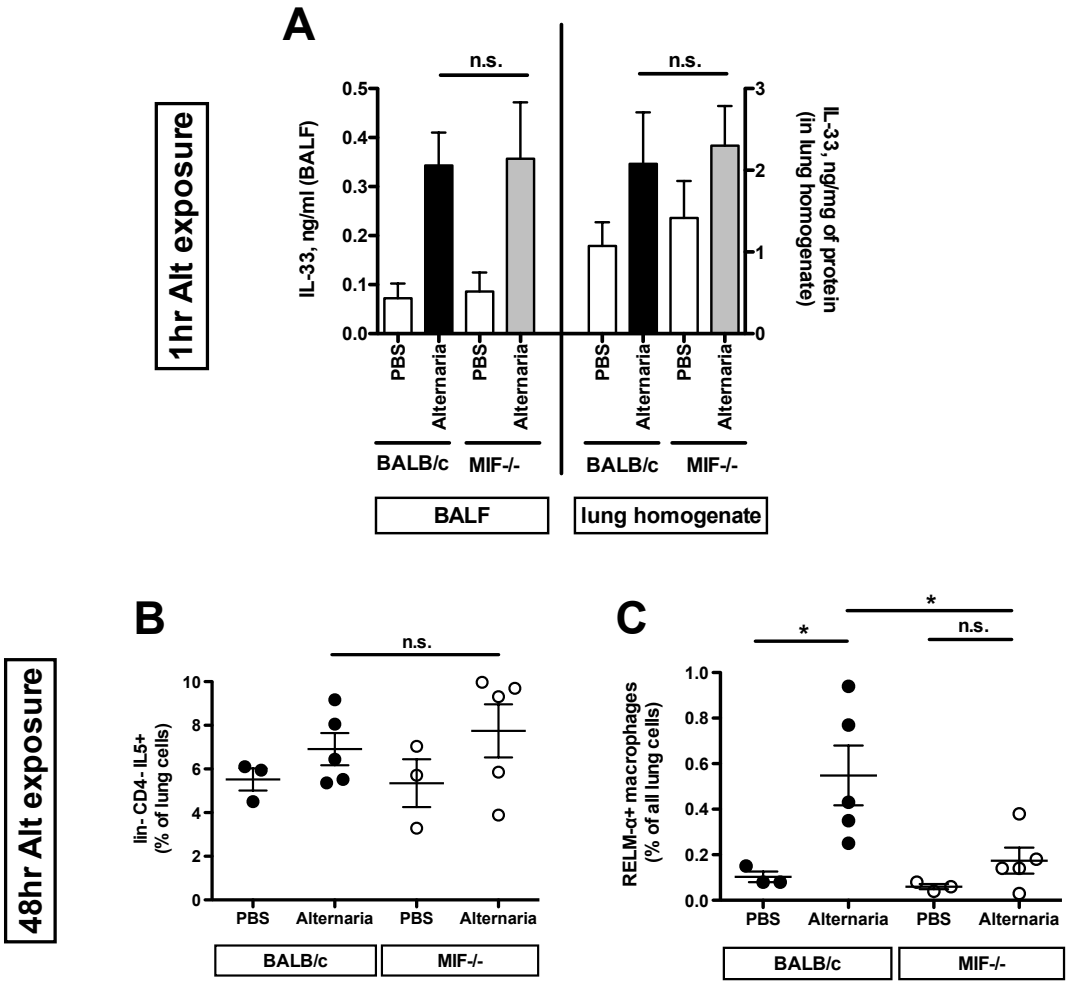


Fig 3.11 - Immune responses in the lung after *Alternaria* antigen administration are comparable between MIF$^{-/-}$ and BALB/c wild-type mice

10 µg of *Alternaria* antigen or PBS were administrated i.n. to BALB/c and MIF$^{-/-}$ mice. BAL and lung homogenate harvested 1 hr after *Alternaria* administration were assessed for IL-33 by ELISA (A). 48 hours after *Alteraria* administration lung tissue was digested as described, and assessed for presence of IL-5+ ILCs (B), and alternatively activated macrophages (RELM-α+ F4/80+ CD11b+)(C). Data represents one experiment per time point. * = p<0.05.

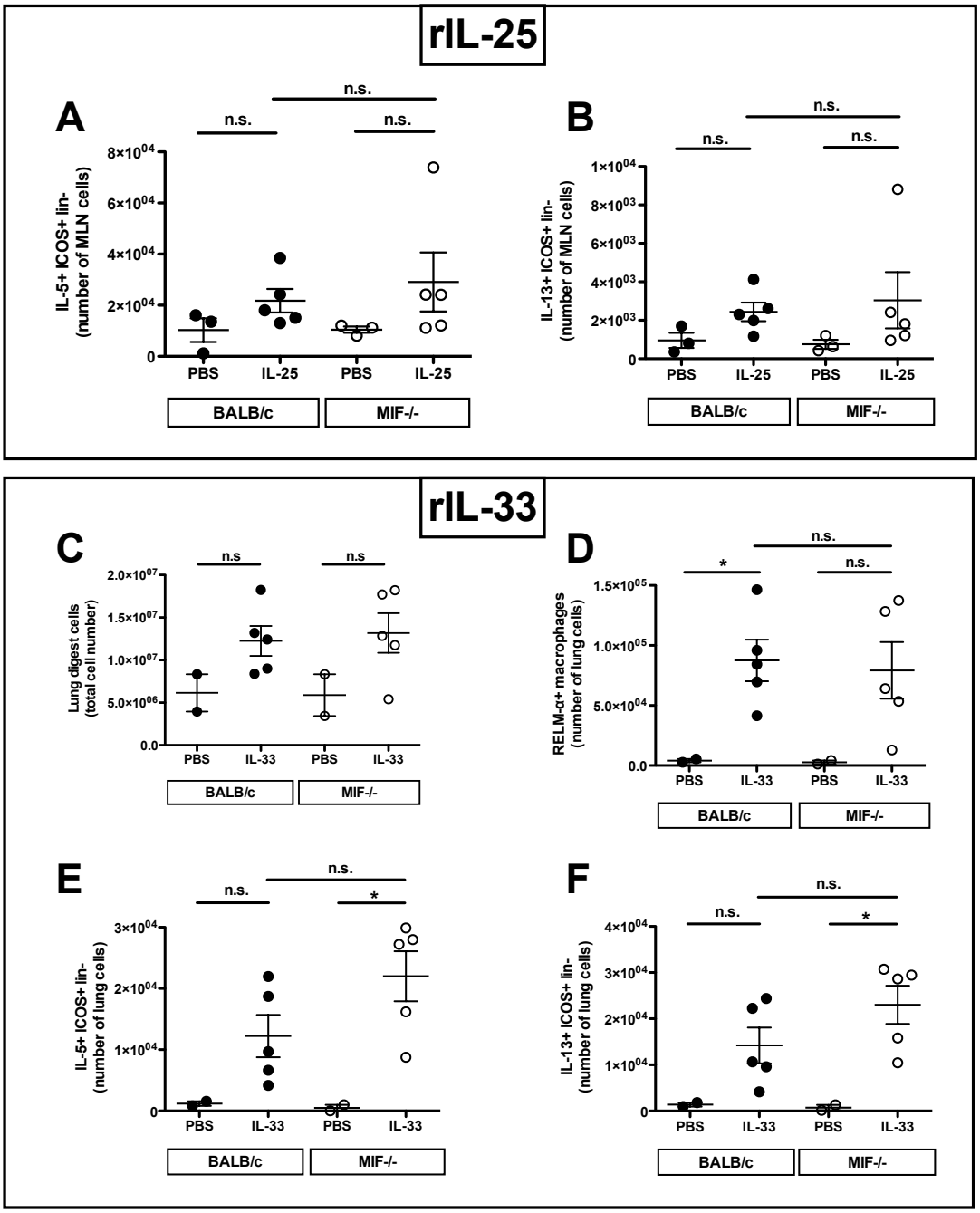


Fig 3.12 - MIF$^{-/-}$ mice can respond to IL-25 and IL-33 to the same extent as BALB/c mice

400 ng of rIL-25 or PBS were administered i.p. to BALB/c and MIF$^{-/-}$ on days 1, 2 and 3. On day 4, MLN cells were assessed for IL-5^{+} ICOS^{+} ILCs (A) and IL-13^{+} ICOS^{+} ILCs (B).
200 ng of rIL-33 or PBS were administered i.n. to BALB/c and MIF$^{-/-}$ on days 1, 2 and 3. On day 4, lung tissue was digested with collagenase, and cells enumerated (C). Cells were assessed for presence of RELM-α F4/80^{+} CD11b^{lo} CD11c^{hi} alveloar macrophages (D), IL-5^{+} ICOS^{+} ILCs (E), and IL-13^{+} ICOS^{+} ILCs (F). .
Data are representative of two independent experiments. *** = p<0.001, ** = p<0.01, * = p<0.05

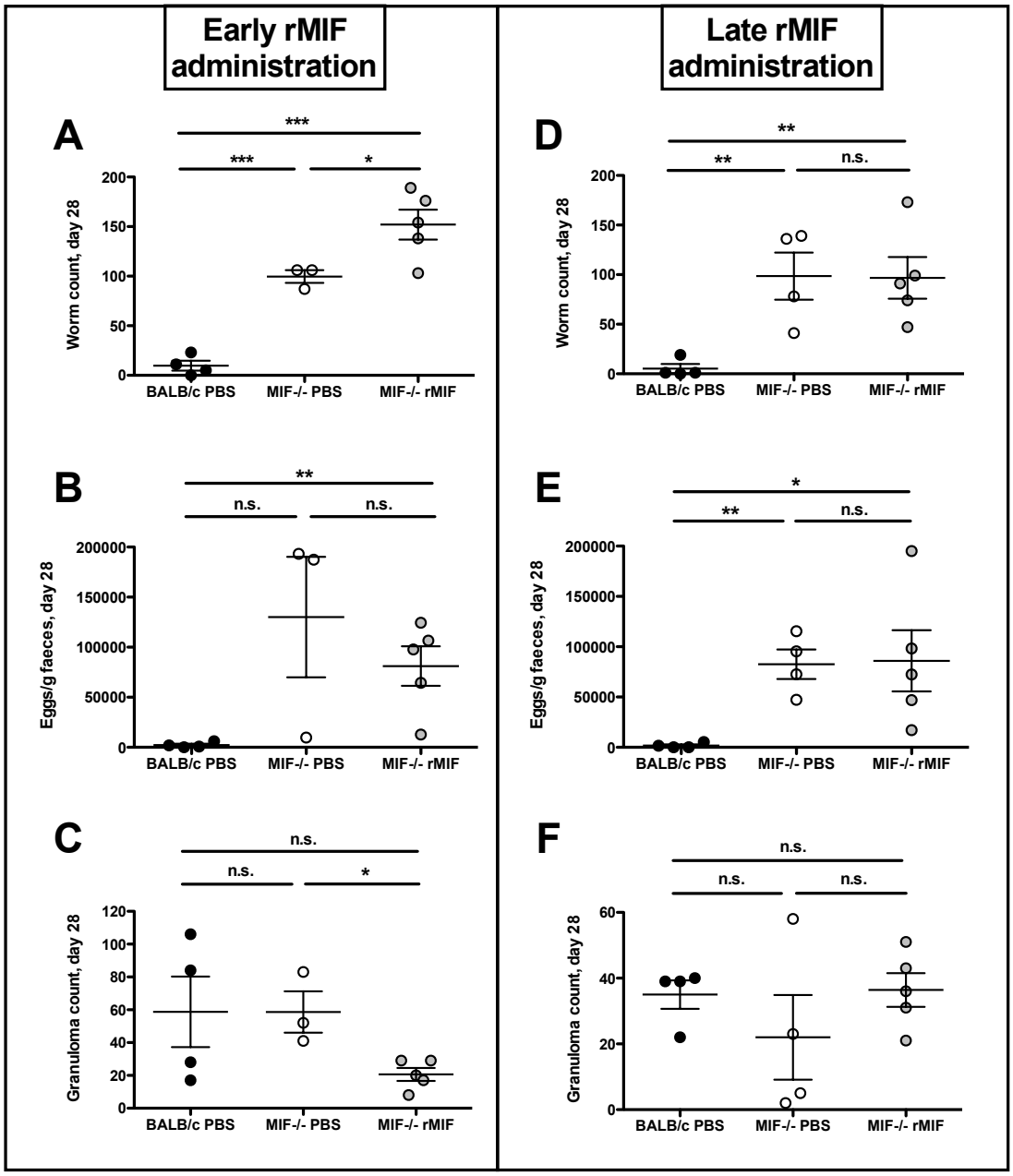


Fig 3.13 - Intra-peritoneal administration of rMIF fails to reverse the MIF$^{-/-}$ phenotype in *H. polygyrus* infection

Female BALB/c and MIF$^{-/-}$ mice were infected with 200 *H. polygyrus* L3 larvae. 50 µg rMIF or PBS was injected i.p. at days -1, 0, 2, 4 and 6 for the "Early" experiment (A-C), and days 14, 16, 18, 20, 22 and 24 for the "Late' experiment (D-F). Adult worm burden, eggs/g faecal material and intestinal granulomas were enumerated at day 28 post-infection for both experiments. Data represent one experiment for each protocol.
*** = p<0.001, ** = p<0.01, * = p<0.05

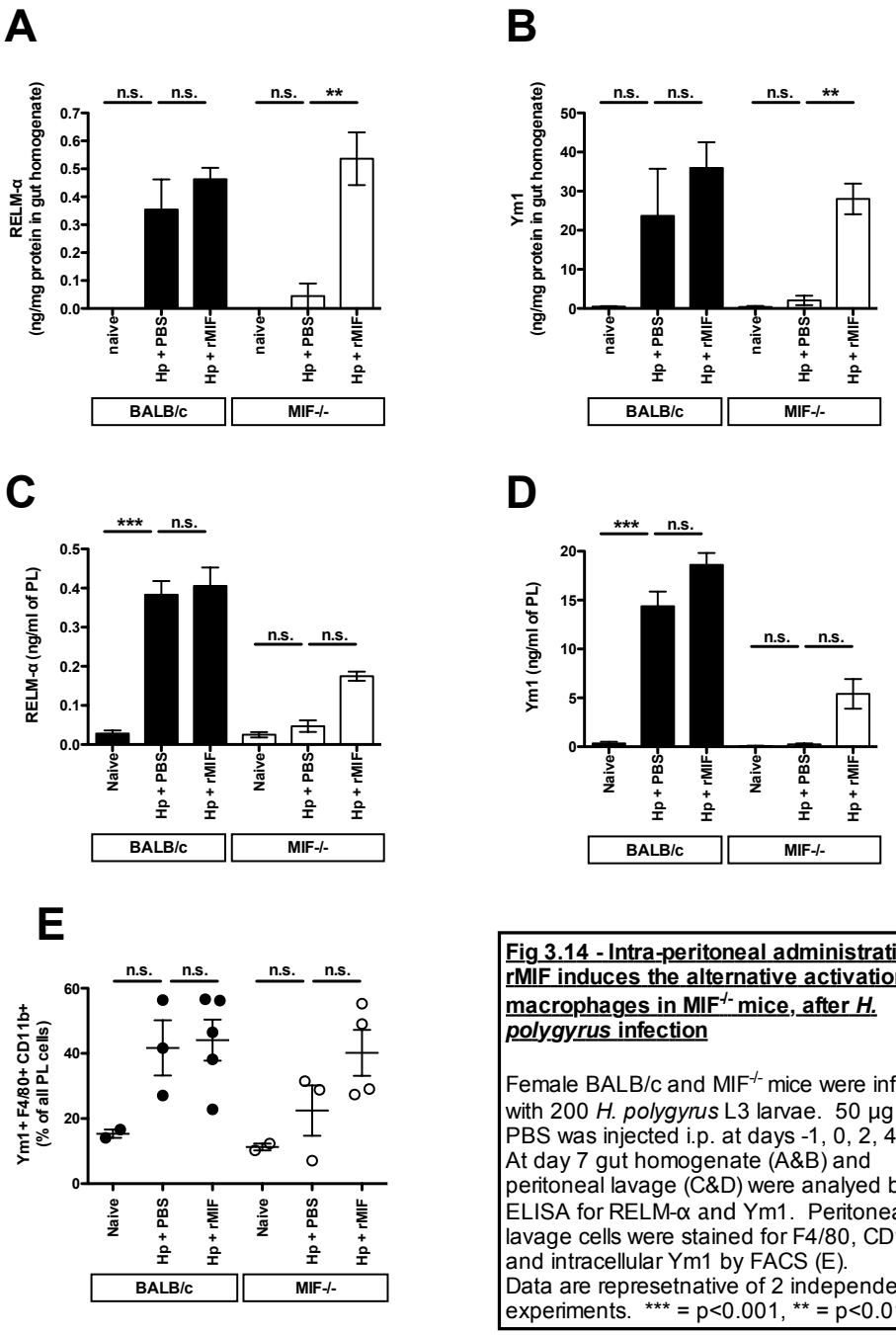


Fig 3.14 - Intra-peritoneal administration of rMIF induces the alternative activation of macrophages in MIF$^{-/-}$ mice, after *H. polygyrus* infection

Female BALB/c and MIF$^{-/-}$ mice were infected with 200 *H. polygyrus* L3 larvae. 50 µg rMIF or PBS was injected i.p. at days -1, 0, 2, 4 and 6. At day 7 gut homogenate (A&B) and peritoneal lavage (C&D) were analyed by ELISA for RELM-α and Ym1. Peritoneal lavage cells were stained for F4/80, CD11b and intracellular Ym1 by FACS (E).
Data are represetnative of 2 independent experiments. *** = p<0.001, ** = p<0.01

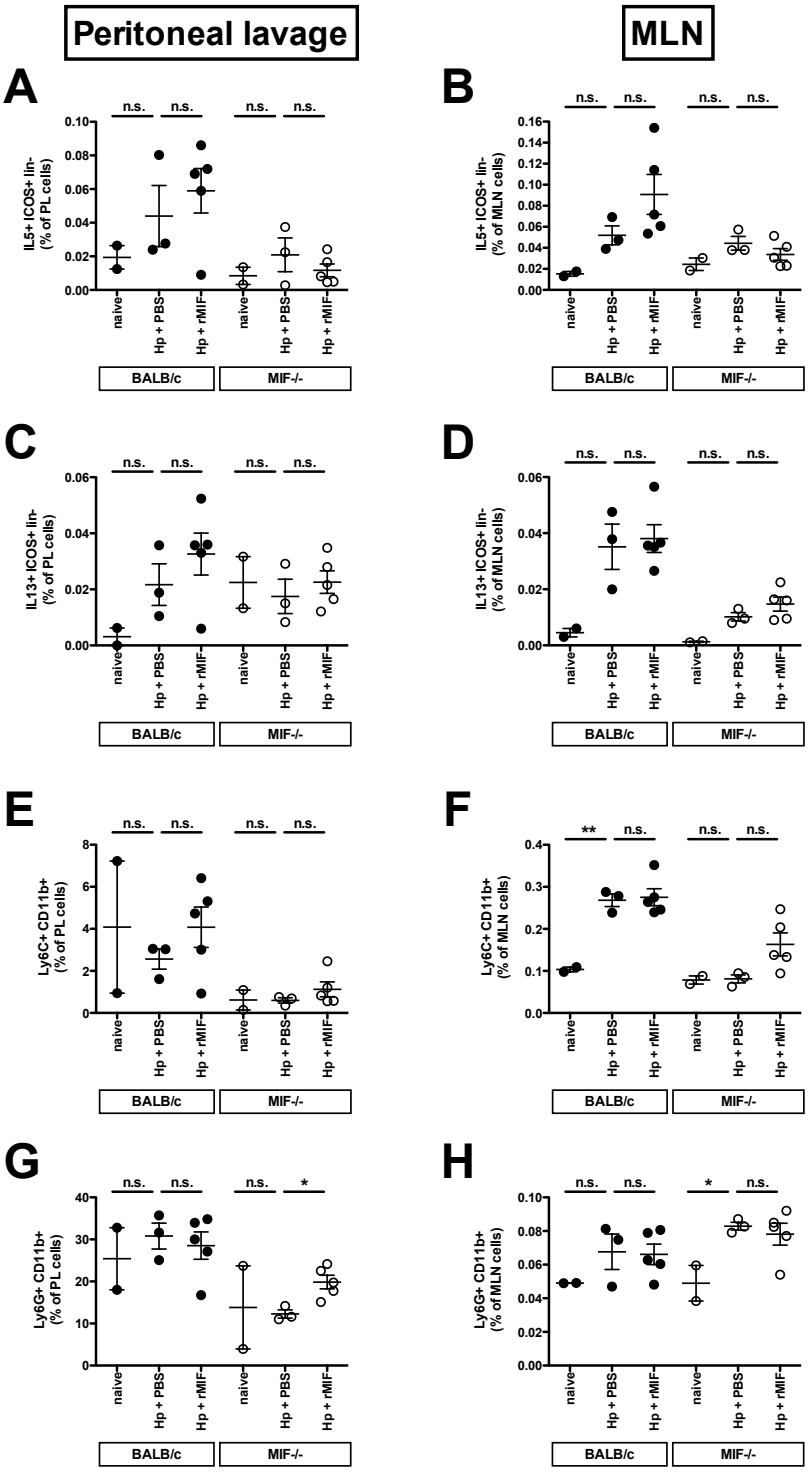


Fig 3.15 - rMIF boosts proportions of ILCs in BALB/c mice and CD11b+ cell populations in MIF$^{-/-}$ mice

Female BALB/c and MIF$^{-/-}$ mice were infected with 200 *H. polygyrus* L3 larvae. 50 µg rMIF or PBS was injected i.p. at days -1, 0, 2, 4 and 6. At day 7, PL and MLN cells were assessed for proportions of IL-5^{+} ICOS^{+} ILCs (A&B), IL-13^{+} ICOS^{+} ILCs (C&D), Ly6C^{+} CD11b^{+} cells (E&F), and Ly6G^{+} CD11b^{+} cells (G&H).
Data are representative of 2 independent experiments. * = $p<0.05$, ** = $p<0.01$.

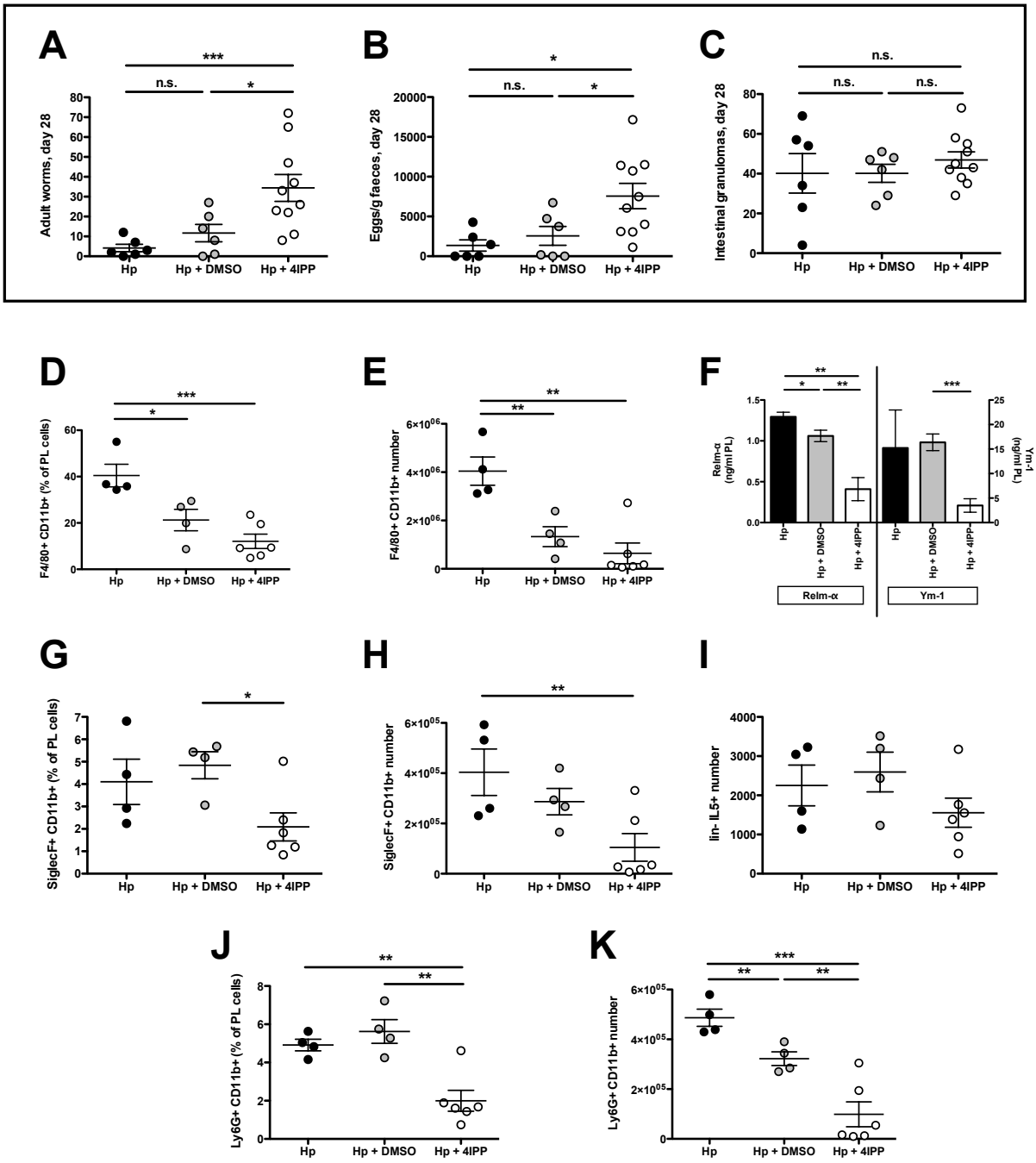


Fig 3.16 - Administration of the MIF chemical inhibitor, 4-IPP, renders BALB/c mice more susceptible to *H. polygyrus*

Female BALB/c mice were infected with 200 *H. polygyrus* L3 larvae. 1 mg 4-IPP in DMSO or DMSO alone was injected i.p. at days -1, 0, 2, 4 and 6 post-infection. Adult worm burden (A), eggs/g faecal material (B) and intestinal granulomas (C) were enumerated at day 28 post-infection. Data are combined from 2 independent experiments.
Using the same protocol, PL cells were analysed, at day 7 post-infection, for proportions (D) and total numbers (E) of macrophages (F4/80$^+$ CD11b$^+$). PL was analysed by ELISA for levels of Relm-α and Ym-1 protein (F). Proportions (G) and total numbers (H) of eosinophils (SiglecF$^+$ CD11b$^+$), the number of IL-5$^+$ ILCs (I), and the proportions (J) and numbers (K) of Ly6G$^+$ CD11b$^+$ cells in the PL were analysed. Data are from one experiment.
*** = p<0.001, ** = p<0.01, * = p<0.05

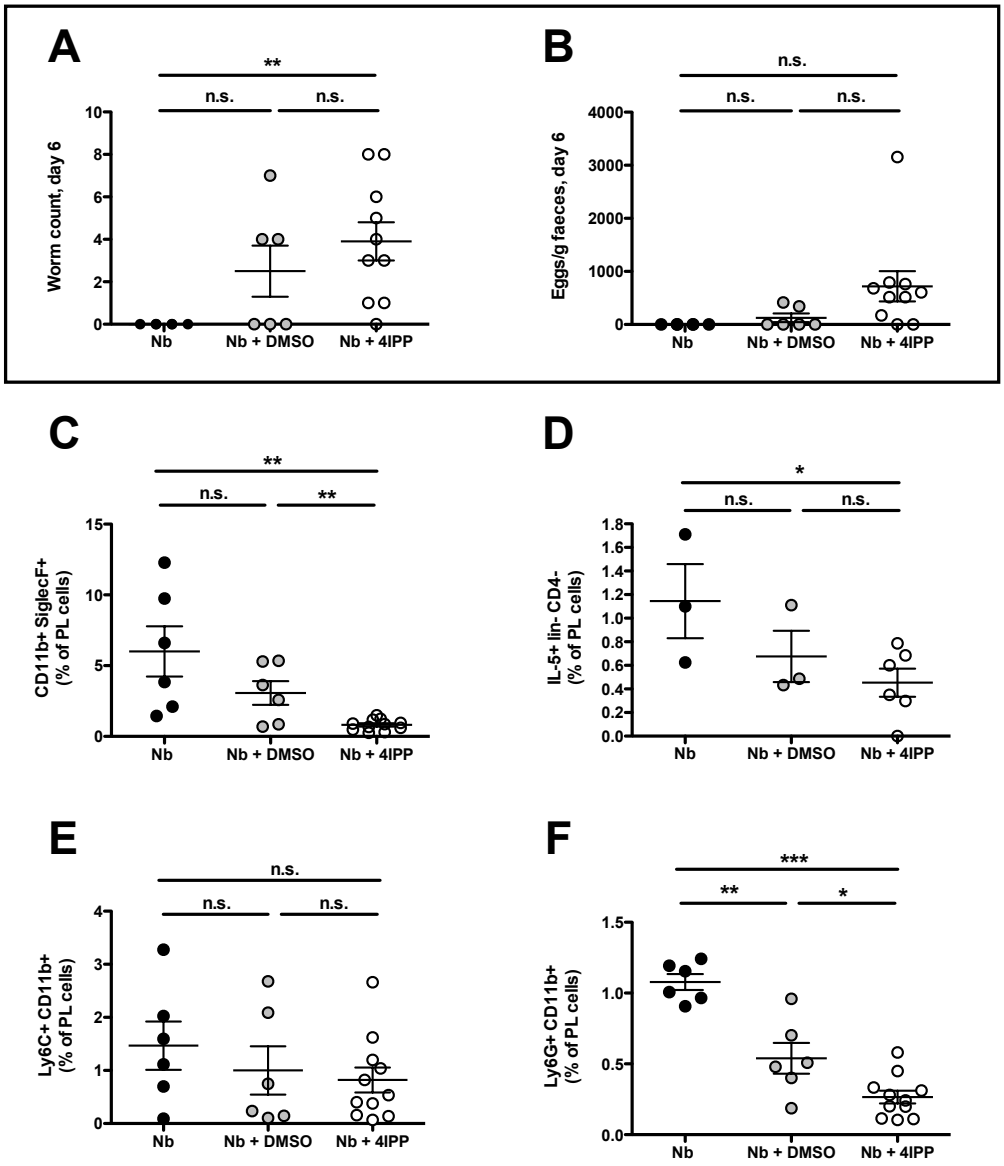


Fig 3.17 - Administration of the MIF chemical inhibitor, 4-IPP, renders BALB/c mice more susceptible to *N. brasiliensis*

Female BALB/c mice were infected with 250 *N. brasiliensis* L3 larvae. 1 mg 4-IPP in DMSO or DMSO alone was injected i.p. at days -1, 0, 2, 4 and 6 post-infection. Adult worm burden (A) and eggs/g faecal material (B) were enumerated at day 6 post-infection. d6 peritoneal lavage cells were analysed by FACS for eosinophils ($CD11b^+$ $SiglecF^+$) (C), ILCs (D), $Ly6C^+$ $CD11b^+$ cells (E), and $Ly6G^+$ $CD11b^+$ cells (F).
The rat strain of *N. brasiliensis* was used throughout. Data are combined from 2 independent experiments, except D which represents one experiment. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Discussion

The importance of MIF in inflammation and innate immunity is well documented (Calandra and Roger 2003). The contribution of this pleiotropic cytokine to autoimmune diseases (Bucala 2013), cancer (Bach *et al* 2008), bacterial infection and sepsis (Calandra *et al* 2000) and immunity to protozoan parasite infection (de Dios Rosado and Rodríguez-Sosa 2011; Bozza *et al* 2012) has been widely investigated. However, the role of MIF in host responses to helminth infections is less well studied (Rodríguez-Sosa *et al* 2003; Magalhães *et al* 2009). Here, the MIF$^{-/-}$ mouse was used to explore the immune responses to *H. polygyrus* and *N. brasiliensis*, 2 widely used gastrointestinal helminth laboratory models.

MIF-deficiency rendered usually resistant BALB/c mice completely susceptible to both parasites, a striking phenotype, which could not be explained by a failure in induction of a strong Th2 response, overproduction of regulatory cells or cytokines, nor, at least in the case of *H. polygyrus*, dysfunctional antibody responses.

Some aspects of innate immunity were defective in MIF$^{-/-}$ mice upon *H. polygyrus* and *N. brailiensis* infections however, including eosinophilia in both MLN and PL. This is a phenomenon noted in the granulomas around *Schistosoma* eggs (Magalhães *et al* 2009) and in allergic asthma (Korsgren *et al* 2000; Magalhães *et al* 2007), indicating a role for maturation and/or migration of eosinophils in an inflammatory setting. *Schistosoma* granuloma numbers mirror the result in *H. polygyrus* in that numbers are similar between wild-type and MIF$^{-/-}$ mice after infection. In the case of *S. mansoni*, the size of granulomas was decreased due to lack of the main cell type, eosinophils, but this had no effect on worm burden or egg output (Magalhães *et al* 2009). Although not formally measured, the size of *H. polygyrus* granulomas does not noticeably change in the MIF-deficient animal, probably because the main cell types here are macrophages and neutrophils (Patel *et al* 2009).

MIF has been linked to the alternative activation of bone marrow-derived macrophages in the presence of IL-4 *in vitro* (Prieto-Lafuente *et al* 2009), and of

tumour-associated macrophages, rendering them less immunosuppressive and inflammatory, leading to exacerbated tumour growth (Yaddanapudi *et al* 2013). It has also been shown to induce the chemotaxis of eosinophils, and the induction of Ym-1 in macrophages when injected *in vivo,* mirroring the effects of live *Brugia malayi* infection (Falcone *et al* 2001). AAMΦ have been described as key cell types in granulomas formed in secondary *H. polygyrus* infection (Patel *et al* 2009), but expression of markers typically associated with these cells has been shown to correlate with resistance to primary *H. polygyrus* in different strains of mice (see chapter 2). $MIF^{-/-}$ mice have similar levels of RELM-α and Ym1 in gut homogenate over the first 2 weeks of infection with *H. polygyrus*, to BALB/c wild-type mice. However, macrophages expressing Ym1 are induced to a lower level in the PL of $MIF^{-/-}$ mice at day 7 post-infection, and rMIF can boost these to levels comparable with BALB/c mice. rMIF also boosts release of RELM-α and Ym1 into the PL and gut tissue of *H. polygyrus* infected BALB/c and $MIF^{-/-}$ mice. Analysis by rt-PCR shows that BALB/c and $MIF^{-/-}$ have comparable levels of message for RELM-α and Ym1, indicating that MIF may be having a downstream effect on the activation or recruitment of AAMΦ to certain inflammatory sites, not on the induction or transcription of key markers by macrophages, or other cell types.

Macrophages have recently been shown to respond to IL-25 and IL-33, cytokines previously attributed to ILC induction (Kurowska-Stolarska *et al* 2009; Yang *et al* 2013b). Although there is no defect in IL-33 production in the lung in $MIF^{-/-}$ mice, and resulting ILC production is intact, the induction of lung AAMΦ is less profound than in BALB/c mice. IL-25 and IL-33 are also made in comparable levels in the gut, as assessed by rt-PCR. This suggests that the effect of MIF-deficiency on proportions of AAMΦ is direct, and does not work through an IL-33-dependent mechanism. Further work could be directed at looking at the roles of both IL-25 and IL-33 in the induction of macrophages in the intestine in *H. polygyrus* infection.

Immunohistochemical analysis of MIF shows more intense staining at day 6, in the early forming granuloma, over day 14, in BALB/c mice, which could indicate a role in the chemoattraction of cells to the inflammatory site by MIF – a role widely

attributed to this cytokine. Future work could strive to dissect the role of MIF in the formation of the granuloma, by using fluorescent microscopy to stain cells in sections from BALB/c versus MIF-/- intestines to assess differences in composition. Attempts to digest live cells from granulomas for FACS analysis and rt-PCR were unsuccessful (data not shown). Optimisation of a method to extract all granuloma cells for FACS or PCR analysis would be ideal, however the most unbiased and exhaustive solution would be profiling whole granuloma gene expression on a microarray.

The function of granulomas in *H. polygyrus* infection is, therfore, not completely clear. Whatever the role of MIF in their formation and function, it is clear that other roles for MIF are important as MIF-deficiency also has profound effects on the outcome of infection with *N. brasiliensis*, a helminth parasite with a very different life cycle. Its infection route, migration to the intestine via the lung, and acute pattern of infection offer a completely different model to the entirely enteric and often chronic nature of *H. polygyrus*. Yet $MIF^{-/-}$ mice are much slower to expel *N. brasiliensis* than BALB/c mice and display immune defects similar to those seen with *H. polygyrus*.

AAMΦ are an important cell type in the lung during *N. brasiliensis* infection, especially with respect to appropriate wound healing, as larval migration causes significant damage to lung tissue (Reece *et al* 2006). Expression of Ym1 and RELM-α in the lung peaks at days 5-7 post-infection (Hoeve *et al* 2009), which is noted in both the BALB/c and $MIF^{-/-}$ mice. Although no defect in the expression of these molecules was apparent, it will be important to confirm where they are being expressed. IHC, co-localisation fluorescent microscopy and further FACS analysis of cells in the lung could show if expression by epithelial cells or another cell type is maintaining equal levels in BALB/c and $MIF^{-/-}$ mice, and if there is comparable wound healing in the lungs. Lung macrophages are thought to act in the killing of *N. brasiliensis* larvae in the lung (Egwang *et al* 1984), and assessing further whether such cells are absent or defective in the $MIF^{-/-}$ setting would be of importance.

In the intestine, AAMΦ and Arg-1 are thought to contribute to the hyper-contractility of the intestinal smooth muscle, which in turn contributes to *N. brasiliensis* adult worm expulsion from the gut (Zhao *et al* 2008). Although no defect was found in the expression of RELM-α and Ym1 in the gut homogenate, future work could assess the macrophage content and phenotype in gut tissue in $MIF^{-/-}$ mice compared to BALB/c, to ascertain if a deficiency of this cell type correlates with an inability to expel the worm.

Type 2 ILCs have only recently been recognized as important initiators of Th2 responses in response to helminth infection and allergic inflammation (Oliphant *et al* 2011) and their specific role in *H. polygyrus* infection has yet to be established. IL-5 producing ILCs are induced by *H. polygyrus* infection in the MLN of BALB/c mice, and this induction is absent in $MIF^{-/-}$ mice. $MIF^{-/-}$ mice show no defect in $IL\text{-}5^{+}$ ILCs when directly stimulated with IL-25 or IL-33. Moreover, ILC2s are induced upon *N. brasiliensis* infection in $MIF^{-/-}$ mice. This may indicate either that in *N. brasiliensis* infection, other factors induced by the worm act redundantly with MIF, and/or that *H. polygyrus* suppresses such factors so that ILC2 induction becomes fully MIF-dependent.

A further possibility is that ILC2 induction may be related to tissue damage, with, for example, the release of MIF from epithelial cells upon migration of *H. polygyrus* through the mucosa of the small intestine. MIF is stored pre-formed in intracellular pools in many cell types, including the intestinal and lung epithelium (Calandra and Roger 2003) and so is poised to be released and act quickly in such a situation, as has been demonstrated in models of liver injury (Liu *et al* 2010a). IL-25, IL-33 and TSLP are thought to act as alarmins upon damage or assault of epithelial cell layers (Saenz *et al* 2008) and MIF could also be acting as an alarmin to induce ILCs when barriers are compromised. It would be vital, therefore, to test if MIF is released in the lung of *N. brasiliensis*-infected animals, as this is where most tissue damage occurs in this model, and from the gut epithelium in *H. polygyrus* infection. Also, to test if MIF is released as an alarmin, the use of cultured epithelial cell monolayers could be utilized, to test if application of parasite products or damage, induces the

quick release of MIF into the environment. A microarray of intestinal epithelial cells from naïve versus infected BALB/c and $MIF^{-/-}$ mice may also elucidate the mechanisms involved.

The induction of cells of both $CD11b^{+}$ $Ly6C^{+}$ and $CD11b^{+}$ $Ly6G^{+}$ phenotypes was found to be significantly lower in both *H. polygyrus* and *N. brasiliensis* infection settings. Inhibition of MIF significantly reduces proportions of $Ly6G^{+}$ cells in the PL of *N. brasiliensis*-infected BALB/c mice, and administration of rMIF can boost proportions of these cells in MIF-deficient mice infected with *H. polygyrus*. These data point to a direct role of MIF in the induction of $Ly6G^{+}$ $CD11b^{+}$ cells during helminth infection. These markers are expressed by several different myeloid-cell types, and as yet it remains unclear which cell populations they represent.

Among cells that display the $Ly6G^{+}$ $CD11b^{+}$ phenotype are granulocytic MDSC, as well as neutrophils, and distinguishing between these populations requires characterization of their *in vitro* suppressive function (Brandau *et al* 2013; Pillay *et al* 2013). Indeed, many of the same transcriptional pathways are upregulated in neutrophils and granulocytic MDSCs (Fridlender *et al* 2012). It is interesting to note that neither MDSCs nor neutrophils have been widely studied in relation to helminth parasite immunity. In *N. brasiliensis* infection, depletion of $Gr1^{+}$ cells results in delayed worm clearance, although the authors ascribed this effect to greater persistence of bacteria that inhibited Th2 responsiveness (Pesce *et al* 2008). $Gr1^{+}$ neutrophils were also found to be the most abundant cell type in primary *H. polygyrus* granulomas by fluorescent microscopy (Anthony *et al* 2006) but in this system have not been attributed any role in parasite killing or clearance. The release of major basic protein from neutrophils was necessary for protective immunity against *Strongyloides stercoralis*, but the presence of eosinophils was also required (Galioto *et al* 2006; O'Connell *et al* 2011).

Interestingly, MIF has been shown to contribute to the chemotaxis of neutrophils to inflammatory sites (Santos *et al* 2011), which could explain a defect in cells with a neutrophilic phenotype in $MIF^{-/-}$ mice upon infection. One study has linked the

depletion of MDSCs with increased susceptibility to *N. brasiliensis*, and the adoptive transfer of the $Ly6G^+$ subset of these worked to control egg production by these worms, whereas the $Ly6C^+$ population did not (Saleem *et al* 2012). The suppressive and protective actions of MDSCs were dependent on the interaction with mast cells in this study. However, in *H. polygyrus* infection, at the d14 time point analysed, $MIF^{-/-}$ had no defect in mast cell induction in the small intestine, suggesting that in this setting, the defective induction of $Ly6G^+$ and $Ly6C^+$ myeloid populations is not due to a defect in mast cells in the $MIF^{-/-}$ animals.

$Ly6C^+$ $CD11b^+$ cells have been termed monocytic MDSCs in settings including tumours (Youn *et al* 2008; Saleem *et al* 2012), where they have been shown to recruit T regs, favouring tumour growth (Schlecker *et al* 2012) through suppression of anti-tumour immunity, and in acute inflammation models where they have been implicated in promoting would healing (Cuervo *et al* 2011; Saiwai *et al* 2013). Other authors classify cells with this phenotype as inflammatory monocytes (Sunderkotter *et al* 2004), which are recruited to allergic skin sites (Egawa *et al* 2013), inflamed colon (Zigmond *et al* 2012) and central nervous system (Denney *et al* 2012). Notably, a significant proportion of cells called inflammatory monocytes have a suppressive phenotype and could therefore be termed MDSCs (Zhu *et al* 2007). These suppressive monocytes have been shown to have a role in tissue repair after injury (Saiwai *et al* 2013), and in suppression of T cell responses in the spleen during *S. mansoni* infection (Marshall *et al* 2001) and EAE (Zhu *et al* 2007). Also, cells bearing the CD11b and Ly6C markers have been found to show functional plasticity dependent on the local inflammatory or tumour microenvironment (Källberg *et al* 2012).

Monocytic MDSCs show a similar activation spectrum to macrophages in becoming skewed to an alternatively activated phenotype in the presence of Th2 cytokines (Ghassabeh *et al* 2006; Umemura *et al* 2008; Yang *et al* 2013a), and accumulate in the peritoneal cavity of mice infected with the helminth *Taenia crassiceps* (Brys *et al* 2005). Both $Ly6G^+$ and $Ly6C^+$ cells utilize L-arginine for their metabolism with the use of enzymes Arg1 and iNOS (Gabrilovich and Nagaraj 2009). It has been shown

that the depletion of arginine in the cellular microenvironment by the metabolism of MDSCs, and the release of reactive oxygen species (ROS), leads to suppression of T cells (Gabrilovich and Nagaraj 2009). In theory, MDSCs could be having the same effect in the larval helminth microenvironment. In terms of *H. polygyrus*, it is unclear whether $Gr1^+$ cells in the granuloma (Anthony *et al* 2006) are all neutrophils, or if suppressive myeloid cells are also present. If so, they could be depleting the environment of amino acids vital to parasite growth and health, and directly damaging larval cuticles with ROS. Much more investigation into this theory is needed, but is an intriguing possibility nonetheless.

Without further phenotyping of the $CD11b^+$ cell subsets it is difficult to attribute a definite name and function to them in relation to MIF and helminth immunity. Isolation of cells with different cell surface markers could be undertaken in different organs after *H. polygyrus* or *N. brasiliensis* infection, cell suppression assays and gene arrays could be performed for further characterization, and culture of bone marrow with rMIF or helminth products could elucidate a role for these factors in the development and induction of different myeloid cell types. A preliminary but intriguing finding is that treatment of BALB/c mice with the anti-Ly6G antibody 1A8 significantly impairs *H. polygyrus* adult worm clearance (J. Hewitson, unpublished data), giving further evidence for the important role of this myeloid cell type in helminth infection.

The 4-IPP experiments showed that MIF could effectively be inhibited in BALB/c mice, for the most part, replicating both parasitological and cellular effects of MIF deficiency. The effect of DMSO on these parameters however, would suggest a different approach could be taken to get even clearer results. Another MIF antagonist commercially available, ISO-1 (Al-Abed *et al* 2005), also uses DMSO as a vehicle, however, a recently described allosteric small molecule p425, does not need DMSO to dissolve, and inhibits MIF by binding to a site outside the catalytic domain (Bai *et al* 2012). This unique mode of action would be interesting to study in the context of helminth infection, and may resolve the issues with DMSO found in these experiments.

Notwithstanding the vehicle effects, MIF has been shown to be a vital component of immunity against 2 very different parasitic helminths in the murine host. It may have direct and indirect effects on key cellular populations that help control parasite fecundity and survival. Future work could focus on the function of MIF as an alarmin, by utilizing epithelial cell culture systems to assess if MIF is released upon damage or application of parasite products. Comparison of genes upregulated in intestinal tissue, upon infection of BALB/c versus $MIF^{-/-}$ by microarray, could elucidate the pathways and mechanisms MIF is involved in. The same approach looking at specific isolated populations of cells from the intestine, MLN or PL could indicate defective signaling pathways or activation statuses of cells when MIF is absent. Although the focus has been on cells already shown to have some link with MIF (eosinophils, macrophages, MDSCs), other cell types may be important in MIFs role in immunity to helminths, and a more global, systematic approach may uncover new links.

The exploration of the potential of MIF as a therapeutic target in inflammatory diseases, and cancer, is well underway (Garai and Lóránd 2009; Adamali *et al* 2012; Zheng *et al* 2012), but further investigation into this complex cytokine in helminth infection may lead to amelioration of immune pathology and chronic inflammation often associated with these parasites. Furthermore, the exploitation of helminth-derived MIF has been shown experimentally to modulate immune responses and inflammation in several models (Amano *et al* 2007; Park *et al* 2009) and could be further exploited to this end.

<u>Key Findings</u>

- MIF deficiency renders normally fast responding BALB/c mice completely susceptible to primary infections with *H. polygyrus* and *N. brasiliensis,* a striking and novel finding.
- In both infection settings, no significant defect in Th2 cytokine responses was found in $MIF^{-/-}$ mice, and in *H. polygyrus* infection, antibody, Treg and frequency of granulomas were comparable to wild-type mice, although MIF

expression was found to be intense in the forming granuloma of MIF-sufficient mice at day 6 post-*H. polygyrus* infection..

- Several populations of innate cells were found to be deficient to varying degrees in $MIF^{-/-}$ mice after parasite infection compared to wild-type BALB/c mice. These included eosinophils, $CD11b^{+}$ $Ly6C^{+}$ cells, $CD11b^{+}$ $Ly6G^{+}$ cells and ILC2s in both the MLN (and PL in the case of *H. polygyrus*). This is the first time that MIF has been linked to induction of ILC responses.
- MIF-deficient animals can make IL-33 at mucosal surfaces in response to a potent allergen, and respond to both IL-25 and IL-33 by inducing ILCs, suggesting that the deficit in ILCs after *H. polygyrus* infection is specific to the infection setting and not a developmental defect.
- Although the administration of rMIF did not "rescue" $MIF^{-/-}$ mice after *H. polygyrus* infection, it did significantly induce the release of markers of macrophage alternative activation in both intestinal tissue and PL in these mice, and boosted ILC2 proportions in BALB/c mice.
- The MIF inhibitor, 4-IPP, rendered BALB/c mice more susceptible to both *H. polygyrus* and *N. brasiliensis,* and had significant effects on the cellular immunity in response to these parasites.

Chapter 4

Identifying potential immunomodulators in HES

Introduction

Transforming growth factor-β (TGF-β) is a key immunomodulatory molecule and is essential for the protective effects of *H. polygyrus* in models of autoimmune disease (Ince *et al* 2009; Reynolds and Maizels 2012). Proteomic studies of HES did not detect any protein with sequence homology to TGF-β (Hewitson *et al* 2011b; Moreno *et al* 2011). This is somewhat surprising considering HES has potent TGF-β activity and promotes $FoxP3^+$ T reg induction *in vitro* from $FoxP3^-$ splenocytes (Grainger *et al* 2010), as well as *H. polygyrus* infection inducing the expansion and activation of T regs *in vivo* (Finney *et al* 2007). Several important observations were noted about the activity of HES: that it induced FoxP3 expression through the TGF-β pathway and via phosphorylation of SMAD-2/3; it acts directly on T cells – T cells with a dominant-negative form of the TGF-βRII (other cells can still signal through this receptor) can not expand FoxP3 expression in response to HES; the T regs induced by HES *in vitro* can mediate protection in a model of allergic asthma (Grainger *et al* 2010).

A TGF-β homologue was isolated from *H. polygyrus* cDNA using PCR and RACE, and found to be more highly expressed in adult worms over larval forms (McSorley *et al* 2009). The same study also found homologues of TGF-β in *N. brasiliensis*, *H. contortus* and *T circumcincta.* *B. malayi* has also been shown to express TGF-β molecules homologous to human TGF-β (Gomez-Escobar *et al* 2000), but they were not detected in proteomic analysis of the ES from the adult worm (Hewitson *et al* 2008; Moreno and Geary 2008).

Continuing the work already published on the TGF-β activity in HES from our laboratory (Grainger *et al* 2010), HES has been fractionated using gel filtration and anion exchange chromatography and found to have clear TGF-β activity in single fractions from both methods. Mass spectrometry of the positive fractions has lead to

a shortlist of possible candidates being investigated further, in the hope of finding a novel *H. polygyrus* TGF-β homologue, which could be tested as a therapeutic agent in the future.

Results

4.1 The TGF-β bioassay

The assay used throughout this chapter to measure the activity of TGF-β in fractions of HES, utilizes fibroblasts from Tgfb $1^{-/-}$ mice with a reporter plasmid containing TGF-β responsive Smad-binding elements coupled to a secreted alkaline phosphatase (SEAP) reporter gene (Tesseur *et al* 2006). Upon binding of TGF-β to its receptor on MFB-F11 fibroblasts Smad 2 and 3 are phosphorylated and form a complex with Smad 4. This complex translocates into the nucleus and binds to inducible DNA elements, termed CAGA boxes, which have previously been ligated into a SEAP2 plasmid, resulting in the secretion of alkaline phosphatase into the medium (Fig 4.1). Fibroblasts were seeded in 96-well plates and either 50 µl of neat fraction or a standard curve of recombinant human TGF-β (Fig 4.1 B) in low-serum media were added and left for 24 hours before detection with pNPP. This method was found to be more sensitive and specific than other ELISA-based or fluorescent methods (Tesseur *et al* 2006).

4.2 Gel filtration of HES gives highly reproducible results

HES, prepared as previously described (with concentration over a 3000 MW cut-off membrane) was run over a gel filtration Superdex 200 10/30 GL 24 ml column, for high-resolution fractionation based on molecular size. 500 µg of HES was injected in 1 ml of PBS, with the injection step integrated into the fractionation method. This method produces a highly reproducible fractionation profile showing the content of HES separated into various peaks over 24 1ml fractions (Fig 4.2). Proteins eluted earlier in the fractionation are of a higher molecular weight than those eluted at the end, and were detected using both 280nm (blue trace) and 210nm lasers (red trace), which indicate protein and peptide bonds respectively. An additional laser at 254nm was included to detect DNA/RNA content.

Protein standard molecular weights had previously been calibrated on the column, using a Gel Filtration HMW Calibration Kit. This included Blue Dextran, which was

used to calculate the void volume of the column (8 ml). Using this kit, the predicted mean molecular weight of molecules eluted in each fraction were calculated (Fig 4.8).

4.3 Gel filtration of HES results in a single fraction containing TGF-β activity

Protein content and complexity can be seen in 1D silver stained gels of the 24 fractions (Fig 4.3 A). It is noteworthy that the predicted molecular weights of the fractions (Fig 4.2A) were considerably larger than those of proteins separated on a gel. It is known that some VALs have a double-domain structure (Hewitson *et al* 2011b; Cantacessi and Gasser 2012), and other molecules in HES could potentially form larger polymers. Denaturing conditions would have disrupted domain linkages and the molecular weights of bands on a SDS-PAGE gel represent monomers, and therefore give lower measurements than those detected during fractionation.

When each fraction was tested on the TGF-β bioassay, a clear peak of activity was seen in fraction 9 only, with levels enriched over those found in total HES (Fig 4.3 B). This is a striking result considering the most intense band in fraction 9 was also seen in fractions 10 and 11 on the SDS-PAGE gel (Fig 4.3 A). This may indicate there are several molecules of the same size but represented by one band, or that a very potent TGF-β activity can be attributed to a protein of low abundance not necessarily detected by silver staining of the gel.

4.4 Anion exchange fractionation of HES results in a peak of TGF-β activity

A MonoQ 5/50 GL 1 ml column was used to separate HES by molecular charge, using a 1M NaCl salt gradient. The length and number of steps of the gradient (shown in green in Fig 4.4 A) were optimised to show clear separation of peaks whilst still giving a manageable number of fractions to work with. 7 fractions were collected before the gradient started, where a large peak representing unbound molecules (such as those with no charge) can be seen. The main length of the gradient was chosen to run from 0-40% salt (0-0.4 M NaCl) over 40 column volumes (40 ml), as this is the range where most protein peaks are eluted. A second step from

40% to 100% salt (0.4-1M NaCl) over 5 column volumes was added, and then several fractions were collected at a maximum 1M salt concentration to ensure all molecules had been eluted.

500 µg of HES was dialysed into start buffer (20 mM Tris-HCl, pH8), and injected in 1 ml sample volume, which resulted in 61 1ml fractions. Proteins with a low net surface charge are eluted earlier in the gradient, whereas those with higher net charges are eluted as the ionic strength of the buffer increases. All three wavelength traces showed comparable peak patterns (Fig 4.4 A), although the absorbance at 210nm was much higher than those at 254 and 280 (a phenomenon also seen in size fractionation profiles in Fig 4.2). Although most proteins absorb light at 280nm, measuring at this wavelength can underestimate protein content of a sample, as this absorption is due to the presence of aromatic amino acid residues on the surface of the protein. If these are not exposed, or not present, the absorbance at 280nm will be lower than that at 210nm, which measures peptide bonds.

Protein content of the first 53 of anion exchange fractions is shown stained with silver on 1D gels (Fig 4.4 B) – the final 8 fractions showed no detectable protein content (data not shown). Note that the fraction at the beginning of each gradient step was not collected (fractions 8, 48 and 54), and so these were not included in further analysis.

TGF-β activity was again concentrated in one major fraction (17), with an increase in concentration from baseline in the 4 fractions preceding it (Fig 4.4 C). The level of TGF-β activity is 7 ng/ml in fraction 17 of the anion exchange method and just under 1 ng/ml in size fraction 9. This could be explained by variations in batches of HES.

4.5 VAL content of gel filtration fractions

Next, the presence of VAL proteins, previously described as major components in HES (Hewitson *et al* 2011b), were measured in the gel filtration fractions. Utilizing mAbs known to bind to VAL-1, 2 or 4 (as described in Chapter 1), or polyclonal antibodies from rats injected with recombinant VAL-3 protein, the relative amounts

of these VALs were quantified in 24 fractions of HES, by ELISA. Each VAL was associated with only two or three fractions, illustrating the successful molecular size separation, and minimal overlap, achieved by this method (Fig 4.5). VAL-4 was detected in fractions 12 and 13, slightly further through elution than the other three VALs (Fig 4.5 D). This was the only single- domain VAL tested, and therefore represents a smaller molecule than VALs 1-3, which have double SCP domains and possess glycosylated linker regions (Hewitson *et al* 2011b). Only VAL-1 had a profile that overlapped with that of the TGF-β activity, and therefore VALs 2-4 can potentially be excluded from further consideration as the molecule conferring this activity to HES.

4.6 VAL content of anion exchange fractions

The same ELISA strategy was undertaken to assess VAL content of anion exchange fractions 1-61. VAL-1 and 2 were present in a wide range of fractions, peaking at fractions 13-16 for VAL-1 (Fig 4.6 A) and 18-24 for VAL-2 (Fig 4.6 B). This may reflect the large number of variants of VAL-1 and 2 in HES, which have been found to have at least seven and three variants at an amino acid level respectively (Hewitson *et al* 2011b), resulting in a more diverse charge separation than VAL-3 and 4.

VAL-3 and 4 were eluted in more defined fractions - 29-30 and 25-26 respectively (Fig 4.6 C, D). Therefore, both VAL-1 and 2 were present in fraction 17, the fraction with TGF-β activity for the anion exchange method. As VAL-1 is a common factor in both fractions this is the only VAL molecule under consideration as a potential candidate for TGF-β activity.

Table 4.1 summarises both methods of fractionation and includes information on which fractions contain TGF-β activity and VAL-1-4 (by ELISA).

Table 4.1 – summary of size exclusion and anion exchange methods of fractionation of HES

	Size Exclusion (Gel filtration)		Anion Exchange
Column used	Superdex 200 10/30 GL		MonoQ 5/50 GL
Column volume	24ml		1ml
Start buffer	PBS		20 mM Tris-HCl, pH8
Elution buffer	PBS		20 mM Tris-HCl + 1M NaCl, pH8
Fractions	24x1ml		61x1ml
VAL-1	9-11		13-17
VAL-2	6-7		18-24
VAL-3	12-13		29-30
VAL-4	10-11		25-26
TGF-β activity	**9**		**17**

4.7 Proteomic analysis of TGF-β-positive fractions reveals common components

Fraction 9 from the gel filtration and fraction 17 from the anion exchange were analysed using liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Ashton *et al* 2001). We have previously identified 374 proteins in HES using both spots excised from 2D PAGE gels and total HES, using this method (Hewitson *et al* 2011b). Therefore, the database of peptide sequences, and identities already in place from matching with the in-house transcriptomic mRNA database (Harcus *et al*, manuscript in preparation) were used to analyse results from the TGF-β-positive fractions. 55 protein hits were returned from fraction 9 (see table 4.2) and 134 from fraction 17 (see table 4.3), of which 20 were common to both lists (table 4.4).

Tables 4.2 and 4.3 show protein hits in order of a probability-based Mascot score (the probability that a protein is present in the fraction analysed). Table 4.4 shows the 20 proteins common to both fractions after cross-referencing. Proteins were identified first by comparison to an in-house database of proteins in HES, HEX and

ES from *H. polygyrus* L4-stage larvae and eggs. If sequences did not match these, BLAST searches for similarities and conserved domains were then carried out for further information. Tables also include information on peptide coverage for each protein identified.

4.8 Bacterially expressed VALs 1-4 do not possess TGF-β activity

As VALs 1 and 2 were highlighted as common components of fraction 9 and 17, in table 4.4, recombinant versions of these proteins, plus VALs 3 and 4, were measured for TGF-β activity. These proteins were made using a Rosetta-gami bacterial expression protocol and were purified over nickel affinity columns (by Yvonne Harcus). All proteins, including HES as a control, were added at 10 μg/ml with 3 further 10-fold dilutions. None of the VALs displayed any TGF-β activity, compared to the highest concentration of HES, which showed 0.6 ng/ml TGF-β activity (Fig 4.7). From this result, all VAL proteins can be ruled out of further consideration for the TGF-β candidate protein. As the most abundant protein family in HES, it will be important in the future to elucidate the function of VALs in *H. polygyrus* ES materials.

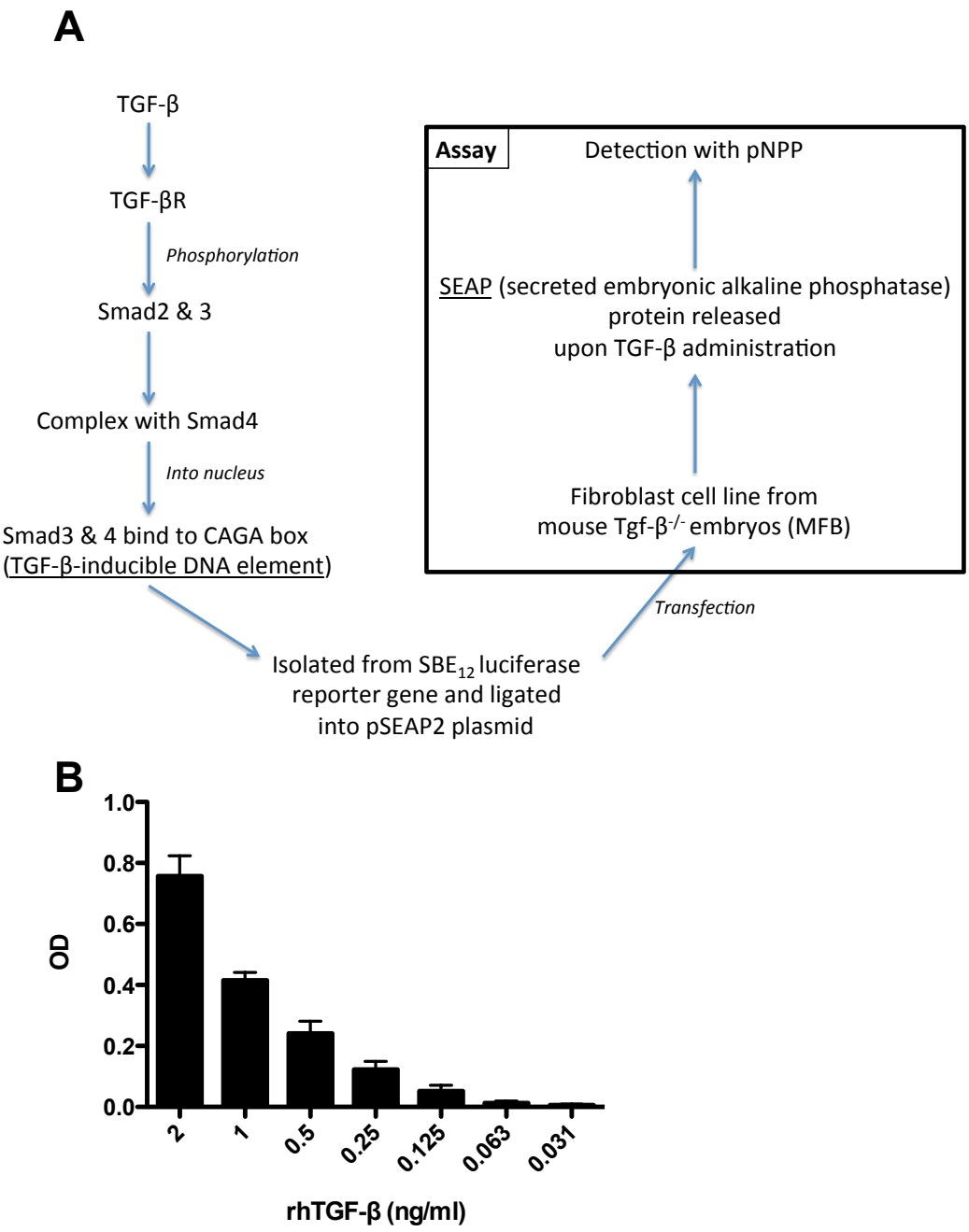
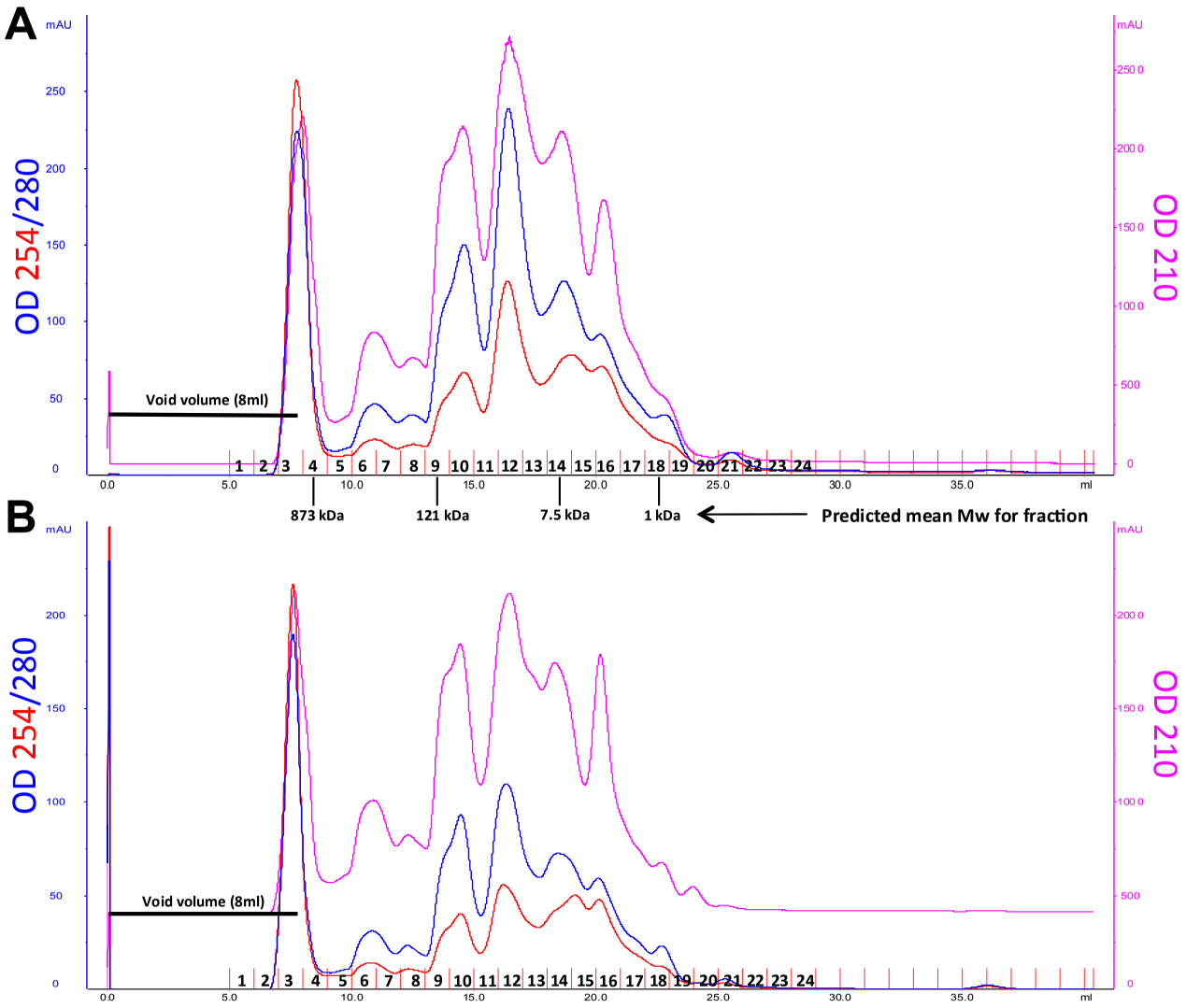


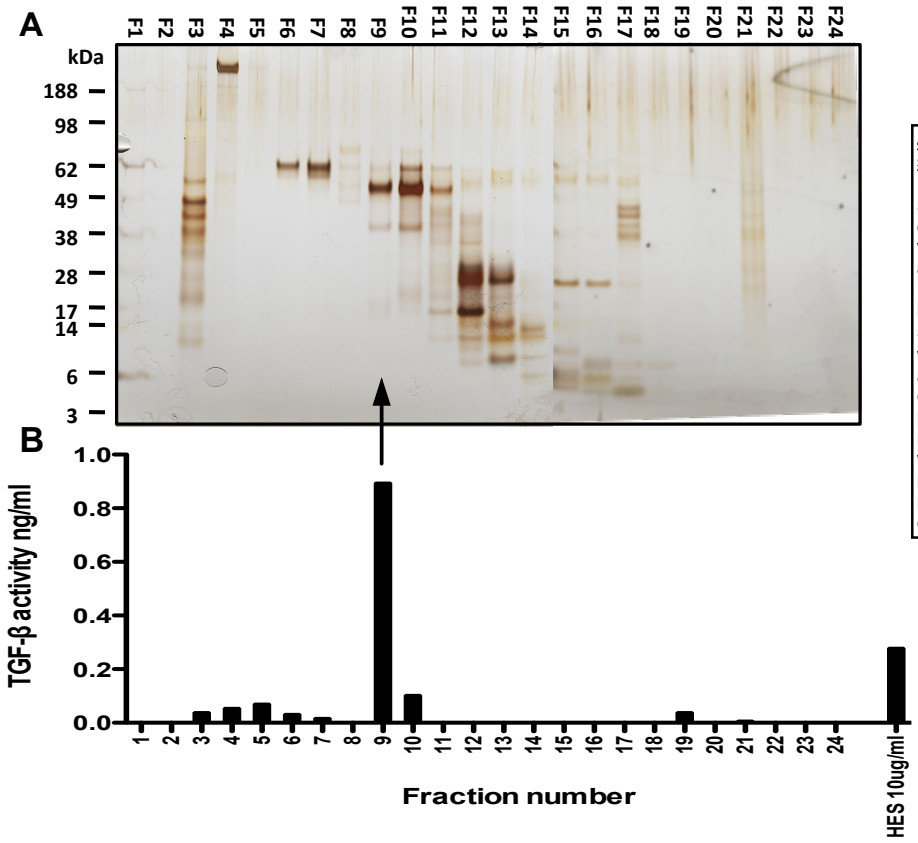
Fig 4.1 The TGF-β bioassay

Overview of TGF-β assay method including development of TGF-β-inducible SEAP secretion by Tgf-$\beta^{-/-}$ MFB-F11 fibroblasts (A)(from (Tesseur et al. 2006)). Serial dilutions of recombinant human TGF-β, starting at 2 ng/ml, were added in 50 µl of DMEM containing 2.5% FCS, to 4 x 10^4 MFB-F11 cells in a 96-well flat-bottomed plate, for 24 hours. Alkaline phosphatase was detected using a SEAP reporter assay kit. Data are representative of several independent experiments.



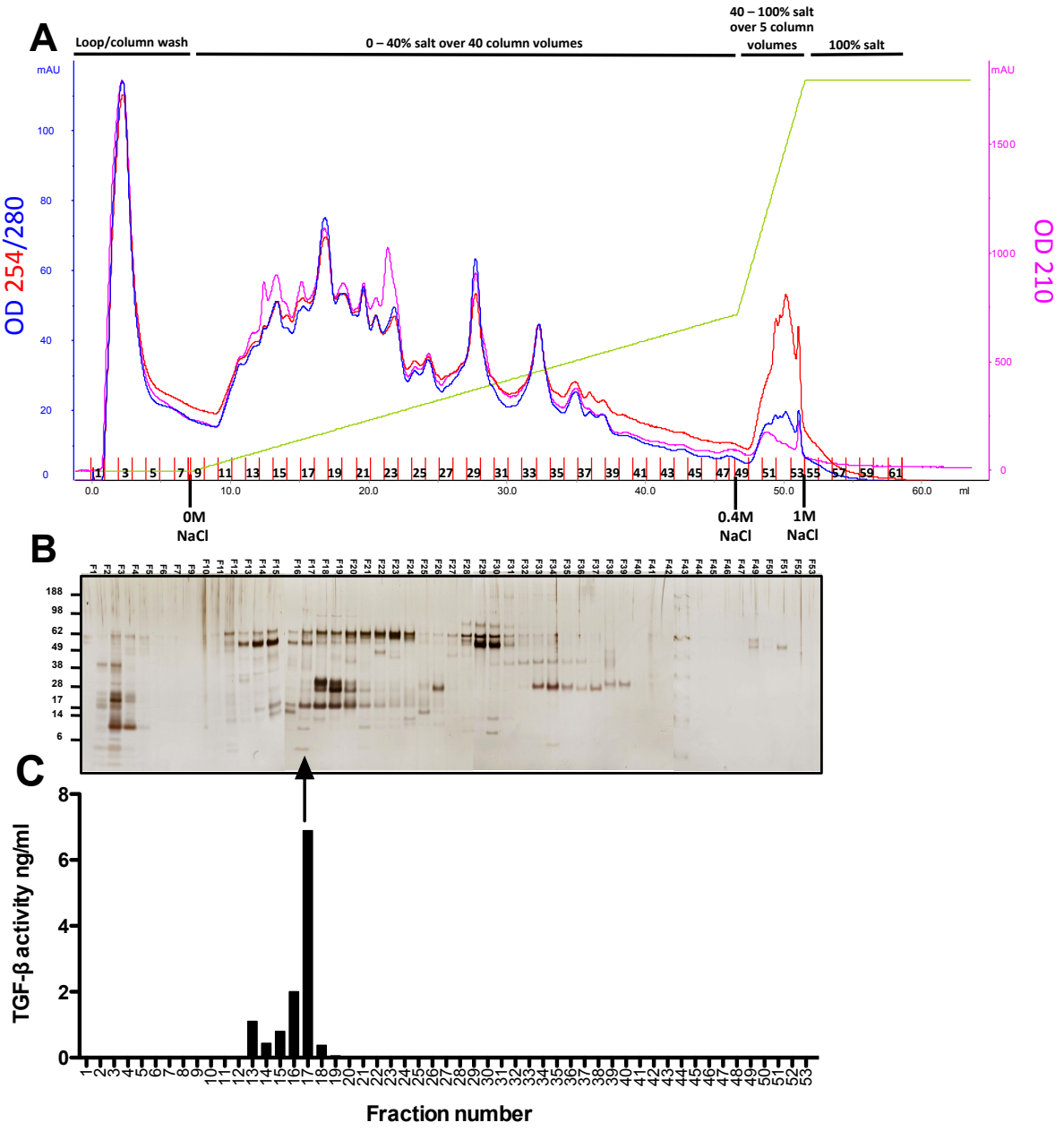
4.2 Gel filtration fractionation of HES gives highly reproducible results

500 µg of HES in 1 ml PBS was run over a gel filtration Superdex 200 10/30 GL 24 ml column, for fractionation based on molecular size. 24 1 ml fractions were collected into a 96 well collection plate using an AKTApurifier system, with a Frac950 fraction collector attachment. Two representative outputs are shown for identical but separate runs, using different batches of HES. Unicorn software was used to track protein content of the fractions by measuring OD 280 (blue) and OD 210 (pink), and RNA/DNA content by OD 254 (red). OD 210 is plotted on right-hand Y axis, with OD 254 and 280 on left-hand Y axis. The 8 ml void volume of the column is indicated. Predicted mean molecular weights for molecules in 4 representative fractions are indicated on panel A. Data are representative of several independent experiments.



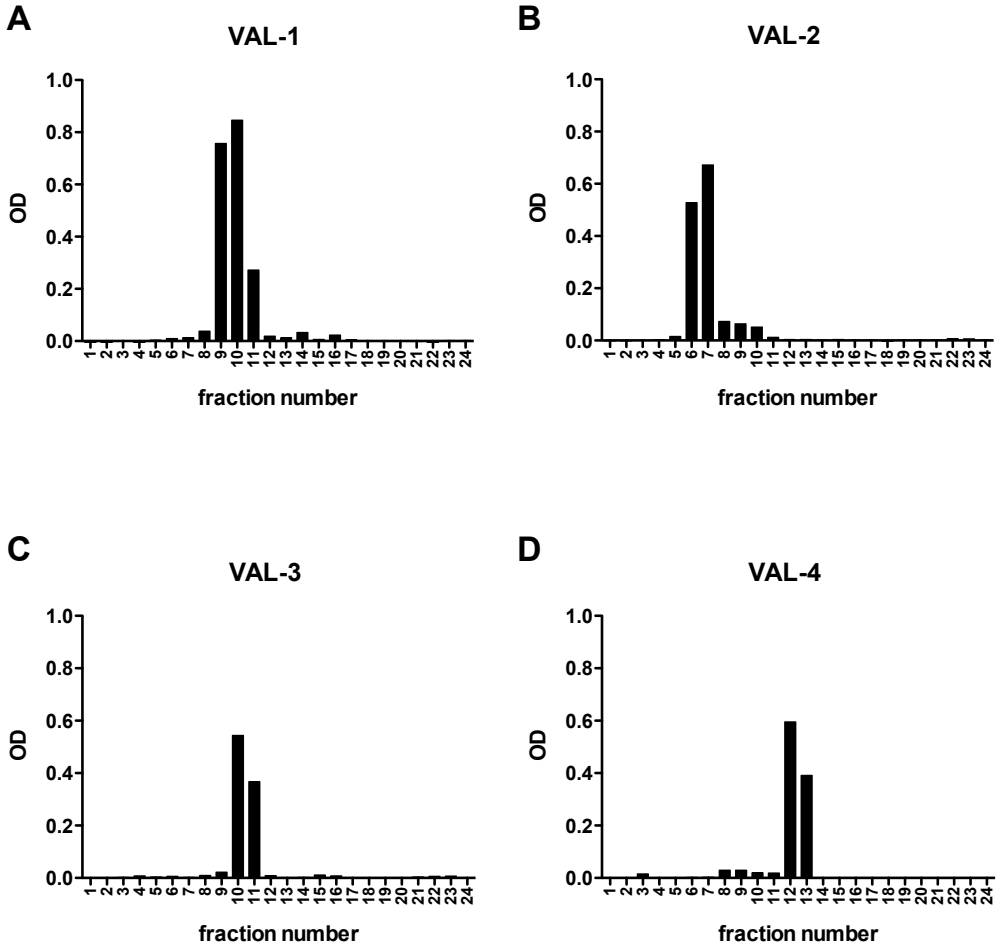
4.3 Gel filtration of HES produces a single fraction containing TGF-β activity

5 µl of each fraction was run on 1 dimensional SDS-PAGE gels, and stained with silver nitrate, to demonstrate complexity and abundance of the proteins.
Ladder is on left hand side and represents kDa (A).
50 µl of each fraction, or HES at 10µg/ml, was incubated with 4 x 10^4 MFB-F11 cells in a 96-well flat bottomed plate, in DMEM containing 2.5% FCS, for 24 hours. Alkaline phosphatase was detected using a SEAP reporter assay kit, and levels of activity were compared to a standard curve of recombinant human-TGF-β (B). Data re representative of several independent experiments.



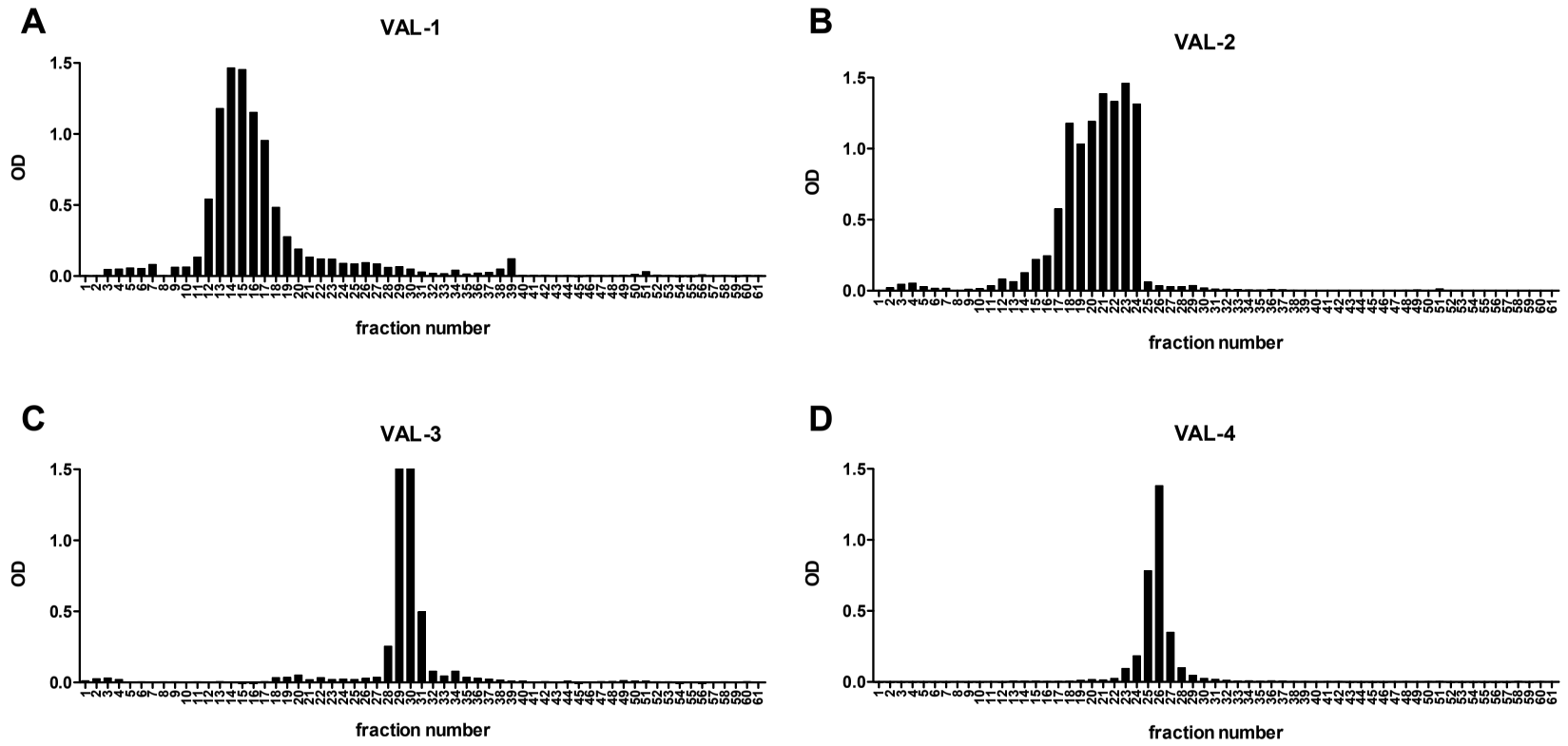
4.4 Anion exchange fractionation of HES results in a peak of TGF-β activity

500 µg of HES in 1 ml 20 mM Tris-HCl, pH8, was run over an anion exchange MonoQ 5/50 GL 1 ml column, for fractionation based on molecular charge. 61 1 ml fractions were collected over a two-step gradient of 20 mM Tris-HCl + 1 M NaCl, pH8, into a 96 well collection plate using an AKTApurifier system from GE Health Care Lifesciences, with a Frac950 fraction collector attachment. Unicorn software was used to track protein content of the fractions by measuring OD 280 (blue) and OD 210 (pink), with RNA/DNA at OD 254 (red). OD 210 is plotted on right-hand Y axis, with OD 254 and 280 on left-hand Y axis. Salt gradient from 0-40% over 40 ml and 40-100% over 5 ml is indicated in green with molar concentrations of NaCl indicated (A).
5 µl of each fraction was run on 1 dimensional SDS-PAGE gels, and stained with silver nitrate, to demonstrate complexity and abundance of the proteins contained in them. Ladder is on left hand side and represents kDa (B).
50 µl of each fraction was incubated with 4 x 10^4 MFB-F11 cells in a 96-well flat bottomed plate, in DMEM containing 2.5% FCS, for 24 hours. Alkaline phosphatase was detected using a SEAP reporter assay kit, and levels of activity were compared to a standard curve of recombinant human-TGF-β (C). Data are represenative of several independent experiments although salt gradients have been modified over time.



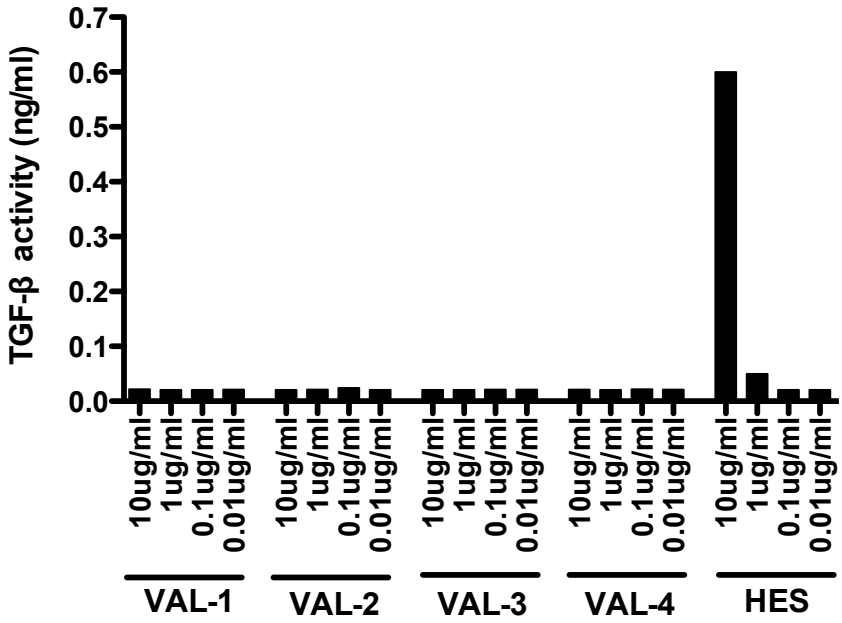
4.5 VAL content of gel filtration fractions

ELISA plates were coated overnight with 50 µl carbonate buffer containing 0.5 µl of a single fraction for each well. Following blocking in TBSt containing 2% BSA, one mAb, or polyclonal rat antibodies, raised against each VAL were used per plate to bind VAL-1, 2, 3 or 4 if present in the fraction (VAL-1 = 4-M15, VAL-2 = 4-S4, VAL-3 = rat pAb, VAL-4 = 2-11). Antibodies were added at 5 µg/ml diluted in 50 µl blocking solution. After incubation at 37° for 2 hours, a secondary anti-mouse IgG1 conjugated to HRP was added at 1/400 dilution for 1 hour, after which detection with ABTS was undertaken. Data are from one experiment.



4.6 VAL content of anion exchange fractions

ELISA plates were coated overnight with 50 µl carbonate buffer containing 0.5 µl of a single fraction for each well. Following blocking in TBSt containing 2% BSA, one mAb, or polyclonal rat antibodies, raised against each VAL were used per plate to bind VAL-1, 2, 3 or 4 if present in the fraction (VAL-1 = 4-M15, VAL-2 = 4-S4, VAL-3 = rat pAb, VAL-4 = 2-11). Antibodies were added at 5 µg/ml diluted in 50 µl blocking solution. After incubation at 37° for 2 hours, a secondary anti-mouse IgG1 conjugated to HRP was added at 1/400 dilution for 1 hour, after which detection with ABTS was undertaken. Data are from one experiment.

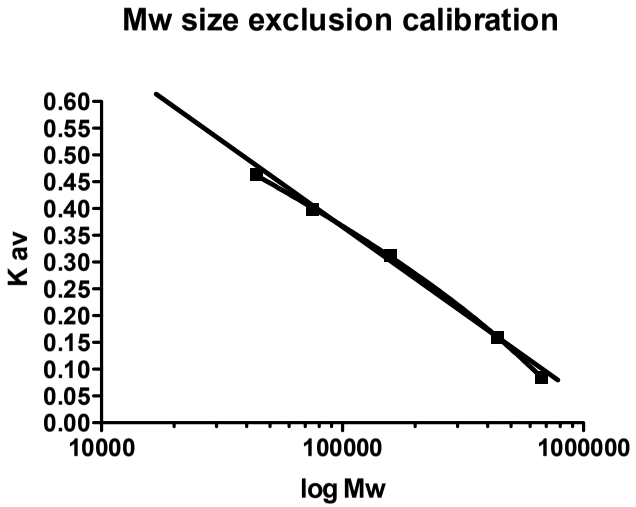


4.7 Bacterially-expressed VALs 1-4 do not posses TGF-β activity

10 µg/ml, 1 µg/ml, 0.1 µg/ml and 0.01 µg/ml of each recombinant VAL protein, or HES, was incubated with 4 x 10^4 MFB-F11 cells in a 96-well flat bottomed plate, in DMEM containing 2.5% FCS, for 24 hours. Alkaline phosphatase was detected using a SEAP reporter assay kit, and levels of activity were compared to a standard curve of recombinant human-TGF-β. Data from one experiment.

Fig 4.8 Calibration of Superdex 200 10/300 GL gel filtration column

6 standard proteins of a known molecular weight were run on the Superdex 200 10/300 GL gel filtration column, at 3 mg/ml, and elution volumes (V_e) for each determined by measuring from the point of injection to the centre of each elution peak. Void volume (V_o) is equal to the elution volume of Blue Dextran, which was run separately at 1 mg/ml. K_{av} values were calculated using the equation as shown with column volume (CV) equal to 24 ml. These were plotted against log Mw to produce a standard calibration curve. K_{av} values were calculated for the mean elution volume for each fraction, and applied to the equation of the calibration curve automatically by Prism software, to produce an estimated mean Mw in kDa for each fraction. The range of the calibration kit spans fractions 4-18.



STANDARDS

Standard protein	Known Mw	Elution peak	Elution volume (V_e)	Void volume (V_o)	K_{av}
	(Da)	(mins)	(ml)		(Ve-Vo/CV-Vo)
Blue Dextran				8ml	
Thyroglobulin	669000	18.69	9.35		0.0841
Ferritin	440000	21.07	10.54		0.1584
Aldolase	158000	25.97	12.99		0.3116
Conalbumin	75000	28.76	14.38		0.3988
Albumin	44000	30.8	15.40		0.4625

CV = column volume = 24ml
Flow rate = 0.5 ml/min
Calibration curve = K_{av} vs log Mw

FRACTIONS

	Elution peak mean	Elution volume mean (V_e)	K_{av}	Approx. Mw	
	(min)	(ml)	(Ve-Vo/CV-Vo)	(Da)	(kDa)
1	11	5.5	-0.1563		
2	13	6.5	-0.0938		
3	15	7.5	-0.0313		
4	17	8.5	0.0313	873030.9	873.0309
5	19	9.5	0.0938	637932.1	637.9321
6	21	10.5	0.1563	449330.2	449.3302
7	23	11.5	0.2188	303660.6	303.6606
8	25	12.5	0.2813	196240.4	196.2404
9	27	13.5	0.3438	121228.9	121.2289
10	29	14.5	0.4063	71907.77	71.90777
11	31	15.5	0.4688	41368.18	41.36818
12	33	16.5	0.5313	23413.02	23.41302
13	35	17.5	0.5938	13233.56	13.23356
14	37	18.5	0.6563	7564.408	7.564408
15	39	19.5	0.7188	4410.044	4.410044
16	41	20.5	0.7813	2634.429	2.634429
17	43	21.5	0.8438	1615.335	1.615335
18	45	22.5	0.9063	1016.577	1.016577
19	47	23.5	0.9688		
20	49	24.5	1.0313		
21	51	25.5	1.0938		
22	53	26.5	1.1563		
23	55	27.5	1.2188		
24	57	28.5	1.2813		

Hp_I14567_IG06511_L1339

BLASTX finds no conserved domains, or identity to any known protein.

A

acaaccacatttatttcattccaagctgatgaaatatccttcatagtgcgcggattccttcctttgtgcaccgatgcacagtt
ttagttccgggatcatgccattcaccgtcagagcactccagctgcaccgttttacctttacattccgcattcactttcgaacc
ggttgcgtagaacctgacgaagtcgtccttataactatcactgccttcttcttctctaaccataactccttcaaagcccagttt
atcaaacaagtcgtcactgttgcaacccccttgcgtttaagcattcgtcaagtgcagtaaaattccactggccgtcaaggca
ttctccgaaaatcggccctttaaccgaacatgtatacggtgacttgtcgcaaaatttttctgacacacgtgtgttgaggggtac
tttccacccttgtctacttccgcgggcgtgccttccttttctgcccgtctcgttggtcatggtgaagtattcgtagtgaacagac
acgttagtttccagtggcttgcatctaggatctgggcatttccttatatgactgtagtaatcccaatgactttcgccagtcgt
accgcttttgtagcaaatgccaacaaattctcctggatcagcttttggctgagtgccctacaacgccttcttgcatgcgttt
gtggagggtaatttccagaactgtccttagatactgctcgtccgacggtatgtcgattgccagcgtatccgtagtattcgta
gaagacaattccatcatctggtagcggtgggcaaccactgggtgggcatgttggtgtgctagaaaaccgccattcagca
ttgtagcacatgccgataatatggccttgaactttgctgtcagcaggaaaatttttgcagattcttttaatgtatgttagttca
ggatattttccagaagcatctggatgaaccgttatattaaaattgattcctgcatttactgtggctttaagatactcgtaggt
gacagtatcgttcgtcggcaacggcgagcacctacggtcttggcattcttgtactccttgatagtagacccattctgatcca
aggcaaatgccgacgtacctacctgtcttttcttctccatgcaatcctttacaaaaccgtttaacatgtgtatgatcaggata
cgctccagagctgtcatttttgtgcaggagtttcgtcgtttctagaacgttctgttaaatatttataggaggcggtttcatcag
aaaatggcatacagccgctggcgtctgtagctgcaacctcgagtaggccaattaatacaatcagcagcatttcatcacgc
taacgataaagggccgttcc

B

3'5' Frame 1

G T A L Y R **Stop** R D E **Met** L L I V L I G L L E V A A T D A S G C **Met** P F S D E T A S Y K Y
L T E R S R N D E T P A Q N D S S G A Y P D H T H V K R F C K G L H G E E K T G R Y V G I
C L G S E W V Y Y Q G V Q E C Q D R R C S P L P T N D T V T Y E Y L K A T V N A G I N F N
I T V H P D A S G K Y P E L T Y I K R I C K N F P A D S K V Q G H I I G **Met** C Y N A E W R F
S S T P T C P P S G C P P L P D D G I V F Y E Y Y G Y A G N R H T V G R A V S K D S S G N
Y P P Q T H A R R R C R A L S Q K A D P G E F V G I C Y K S G T T G E S H W D Y Y S H I R
K C P D P R C K P L E T N V S V H Y E Y F T **Met** T N E T G R K E G T P A E V D K G G K Y P
Q H T C V R K F C D K S P Y T C S V K G P I F G E C L D G Q W N F T A L D E C L N A R G C
N S D D L F D K L G F E G V **Met** V R E E E G S D S Y K D D F V R F Y A T G S K V N A E C K
G K T V Q L E C S D G E W H D P G T K T V H R C T K E G I R A L **Stop** R I F H Q L G **Met**
K **Stop Met** W L

BLUE = peptides from MS in both fractions 9 and 17

C

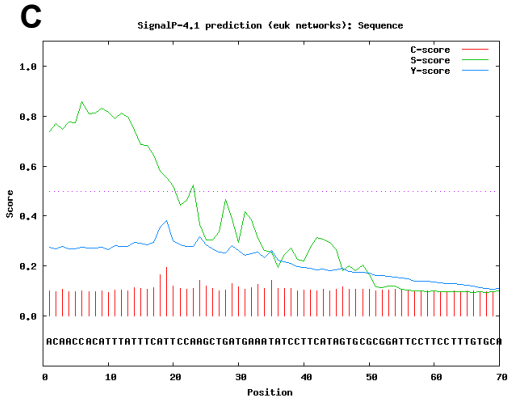


Fig 4.9 Sequence of unknown molecule in Table 4.4

Nucleotide sequence of Hp_I14567_IG06511_L1339 (A), the only protein identified in both fraction 9 and 17 that has not been previously named in *H. polygyrus* databases or found in BLAST searches. BLASTX does not identify any conserved domains. Amino acid in-frame sequence of Hp_I14567_IG06511_L1339 (B). Peptide hits in results from MS of fractions 9 and 17 are indicated in blue.
Analysis on SignalP online software reveals an ambiguous result with regards to the presence of a signal sequence (C). C-score (red bars) indicates signal sequence cleavage sites. S-score (green trace) indicates positions within signal peptides measured from positions in mature parts of the protein. Y-score (blue trace) is the geometric mean of the C-score and slope of the S-score.

Table 4.2

Proteins identified in fraction 9 from the gel filtration fractionation of HES, using LC-MS/MS. Proteins are ranked in order of Mascot score, and number of peptide matches for each protein is included. ID code corresponds to an in-house database of proteins found in adult and larval HES, and ES from eggs, from which the protein identification was assigned.

Rank	Protein identification	ID code	Mascot score	Peptide matches
1	VAL-01.1	Hp_I13898_IG05842_L1539	7424	196
2	VAL-1.2	Hpb-VAL-1.2	5873	149
3	VAL-01.3	Hp_I04202_IG00583_L1521	3234	92
4	Vitellogenin	Hp_I12337_IG04281_L4997	1897	76
5	Vitellogenin	Hp_I01061_IG00070_L5128	1631	59
6	Vitellogenin	Hp_I01065_IG00070_L5127	1520	54
7	VAL-01.5 FRAGMENT	Hp_I22486_IG14430_L629	1061	23
8	VAL-01.4	Hp_I08791_IG02480_L1486	1053	23
9	NSP-006	Hp_I25440_IG17384_L551	847	19
10	VAL-01.7 FRAGMENT	Hp_I17933_IG09877_L867	756	18
11	NSP-004	Hp_I24607_IG16551_L570	620	12
12	VAL-18	Hp_I15283_IG07227_L1200	554	17
13	Zinc metalloprotease-1	Hp_I12336_IG04280_L5030	498	16
14	Zinc metalloprotease-2	Hp_I12444_IG04388_L2875	458	15
15	APY-1.2	Hp_I04667_IG00729_L1917	437	19
16	APY-1.1	Hp_I15979_IG07923_L1089	436	19
17	VAL-2.2	Hpb-VAL-2.2	428	14
18	APY-1.3	Hp_I15931_IG07875_L1098	414	16
19	Aspartyl protease inhibitor	Hp_I15089_IG07033_L1228	396	14
20	NSP-018	Hp_I10136_IG03152_L609	390	11
21	Cathepsin A	Hp_I13848_IG05792_L1547	389	11
22	Legumain	Hp_I02849_IG00289_L1962	386	7
23	(unknown)	Hp_I38562_IG30506_L390	341	9
24	Sushi-like	Hp_I24007_IG15951_L583	325	11
25	VAL-01.6 FRAGMENT	FL8UM6J01DXUGS	323	15
26	CSP-02	Hp_I17532_IG09476_L903	294	8
27	VAL-1.4	Hpb-VAL-14	293	9
28	VAL-09	Hp_I14221_IG06165_L1437	291	11
29	Sushi-like	Hp_I16462_IG08406_L1016	222	7
30	VAL-5	Hpb-VAL-5	207	3
31	Sushi-like	Hp_I24294_IG16238_L579	178	4
32	Poly(U)specific endoribonuclease	Hp_I13201_IG05145_L1858	163	5

33	Aspartyl protease (necepsin)	Hp_I08139_IG02154_L1715	146	5
34	VAL-16	Hp-VAL-16	121	5
35	Aspartyl protease (necepsin)	Hp_C20853_IG04237_L656	117	4
36	VAL 19.1	Hp_I14044_IG05988_L1484	106	2
37	VAL-15	Hpb-VAL-15	93	3
38	Sushi-like	Hp_I12998_IG04942_L2000	93	5
39	Keratin-like protein	Hp_I18857_IG10801_L798	85	2
40	Propylcarboxypeptidase	Hp_I13824_IG05768_L1558	82	4
41	NSP-53.1	Hp_I14567_IG06511_L1339	81	3
42	Apyrase-3	Hp_I06738_IG01430_L1584	71	3
43	C-type lectin	FL8UM6J01B6ULB	70	1
44	CSP-4	GWDWRH002CEQRD	67	3
45	(unknown) (42% ID to NSP-4)	Hp_I26453_IG18397_L531	66	1
46	Chitinase	Hp_I13874_IG05818_L1547	66	2
47	NSP-003.1	Hp_I22470_IG14414_L621	64	2
48	VAL-20	Hp_I07922_IG02045_L1519	54	1
49	NSP-003.2	Hp_I17967_IG09911_L866	40	1
50	CSP-21	Hp_I18812_IG10756_L802	38	1
51	Sushi-like	Hp_I15902_IG07846_L1103	38	1
52	TRP	Hpb-TRP	36	1
53	Sushi-like	Hp_I14129_IG06073_L1461	34	1
54	VAL-3	Hpb-VAL-3	32	1
55	(unknown)	Hp_I16514_IG08458_L1014	31	1

Abbreviations: VAL – Venom Allergen-like protein; APY – apyrase; CSP – conserved secreted protein with signal peptide; NSP – novel secreted protein with signal peptide.

Table 4.3

Proteins identified in fraction 17 from the anion exchange fractionation of HES, using LC-MS/MS. Proteins are ranked in order of Mascot score, and number of peptide matches for each protein is included. ID code corresponds to an in-house database of proteins found in adult and larval HES, and ES from eggs, from which the protein identification was assigned.

Rank	Protein identification	ID code	Mascot score	Peptide matches
1	VAL-2.2	Hpb-VAL-2.2	7424	199
2	VAL-02.2	Hp_I15068_IG07012_L1238	7363	198
3	VAL-02.1	Hp_I07953_IG02060_L1482	6865	183
4	Legumain	Hp_I02849_IG00289_L1962	3579	73
5	Cysteine protease (necpain)	Hp_I15488_IG07432_L1169	2827	88
6	Keratin-like protein	Hp_I10155_IG03162_L783	2553	57
7	Chitinase	Hp_I13874_IG05818_L1547	2248	74
8	15 kDa Hc homologue	Hp_I24441_IG16385_L572	2451	55
9	Myoglobin	Hp_I21133_IG13077_L683	1645	58
10	VAL-8	Hpb-VAL-8	1433	50
11	SXC-like 4	GNK0QLK03GQOZO	1419	55
12	Chondroitin family member	Hp_I05472_IG00957_L1361	1157	36
13	Macrophage migration inhibitory factor (MIF)	Hp_I26227_IG18171_L535	1027	33
14	NSP-005	Hp_I25311_IG17255_L555	918	45
15	VAL-01.7 FRAGMENT	Hp_I17933_IG09877_L867	914	51
16	Myoglobin	Hp_I20188_IG12132_L723	729	24
17	Trypsin family protein	Hp_I15710_IG07654_L1132	672	17
18	Myoglobin	Hp_I03894_IG00508_L604	648	19
19	Vitellogenin	Hp_I01061_IG00070_L5128	608	25
20	Keratin-like protein	Hp_I18857_IG10801_L798	600	22
21	(unknown)	Hp_I17809_IG09753_L879	501	15
22	VAL-01.5 FRAGMENT	Hp_I22486_IG14430_L629	500	24
23	NSP-004	Hp_I24607_IG16551_L570	413	16
24	Astacin protease family member	Hp_I16239_IG08183_L1050	378	13
25	VAL-1.1	Hpb-VAL-1.1	360	18
26	VAL-1.2	Hpb-VAL-1.2	349	18
27	(unknown)	HICN8C106HP9PI	338	10
28	Chondroitin family member	Hp_I17130_IG09074_L942	320	11
29	Metalloprotease/Astaci	Hp_I09071_IG02620_L1210	319	9

	n			
30	VAL-01.4	Hp_I08791_IG02480_L1486	316	20
31	VAL-11	Hp_I01207_IG00080_L1679	311	17
32	Aspartyl protease (necepsin)	Hp_I07646_IG01907_L1927	306	11
33	Glutamine dehydrogenase	Hp_I05693_IG01031_L1691	298	16
34	Myoglobin	Hp_I03893_IG00508_L677	274	9
35	(unknown)	Hp_I20314_IG12258_L716	261	6
36	ERP-13 (only in egg ES)	Hp_I29394_IG21338_L484	257	9
37	NSP-42	HICN8C104EI0NY	239	7
38	Kunitz inhibitor	Hp_I07995_IG02081_L699	234	9
39	Ladder	Hp_I04570_IG00700_L4683	216	6
40	Myoglobin	Hp_I22261_IG14205_L639	215	7
41	(unknown)	Hp_I07468_IG01818_L2128	199	4
42	NSP-006	Hp_I25440_IG17384_L551	194	9
43	Glutamine-6-phosphate isomerase	Hp_C00318_IG00001_L1664	176	6
44	Sushi-like	Hp_I16462_IG08406_L1016	176	3
45	NSP53.1	Hp_I14567_IG06511_L1339	175	14
46	(unknown)	FL8UM6J01DGCZI	174	4
47	Cysteine protease	Hp_I01023_IG00066_L1337	171	9
48	C-type lectin (only in egg ES)	Hp_I10590_IG03379_L588	160	17
49	NSN-006	Hp_I24705_IG16649_L569	153	7
50	DUF148 domain containing protein	Hp_I20153_IG12097_L724	151	8
51	(unknown)	Hp_I01894_IG00147_L383	151	5
52	Lysozyme-6	Hp_I27254_IG19198_L516	148	3
53	VAL-01.3	Hp_I04202_IG00583_L1521	147	5
54	(unknown)	Hp_I10156_IG03162_L593	142	2
55	VAL-07.4	Hp_I01449_IG00104_L991	141	4
56	Vitellogenin	Hp_I12337_IG04281_L4997	136	6
57	Chitin binding Peritrophin-A domain containing protein	Hp_I13075_IG05019_L1949	130	5
58	NSP-002	Hp_I23776_IG15720_L592	125	12
59	Astacin protease family member	Hp_I13832_IG05776_L1555	124	4
60	(unknown)	Hp_I12654_IG04598_L2357	121	2
61	Sushi-like	Hp_I17476_IG09420_L907	120	5
62	Lysozyme-1	Hp_I08665_IG02417_L1321	119	3
63	Myoglobin	Hp_I08959_IG02564_L1212	112	4
64	Catalase (only in egg ES)	Hp_I13437_IG05381_L1722	111	5
65	NSP-001	Hp_I22851_IG14795_L620	107	3

66	CSP-18	Hp_I08021_IG02095_L2057	107	6
67	(unknown)	Hp_I16694_IG08638_L992	100	2
68	VAL-7.1	Hpb-VAL7.1	99	3
69	NSP-053	Hp_I03160_IG00349_L1402	98	6
70	VAL-19.1	Hp_I14044_IG05988_L1484	91	2
71	(unknown) (80% ID to glutamate dehydrogenase from *T. circumcincta*)	Hp_I07874_IG02021_L1689	90	3
72	Sushi-like	Hp_I24007_IG15951_L583	90	7
73	C-type lectin mannose receptor-like (only in egg ES)	Hp_I22655_IG14599_L627	87	5
74	VAL-4	Hpb-VAL-4	86	5
75	Cathepsin B-like cysteine protease	Hp_I14314_IG06258_L1412	85	3
76	Aldolase	Hp_I07682_IG01925_L1479	83	2
77	Lysozyme-5	Hp_I05746_IG01049_L1033	82	2
78	Sushi-like	Hp_I09123_IG02646_L960	80	5
79	NSP-030	Hp_I19157_IG11101_L778	78	3
80	Acid phosphatase	Hp_I13897_IG05841_L1536	76	3
81	NSP-15	GSXTT4C08JWFT8	72	2
82	(unknown)	GLSD98I05FQMBY	71	3
83	MSP-domain containing protein (only in egg ES)	Hp_I19773_IG11717_L745	64	1
84	(unknown)	Hp_I38222_IG30166_L388	64	2
85	SXC-like 6	Hp_I31855_IG23799_L456	63	2
86	VAL-12	Hpb-VAL-12	62	2
87	(unknown)	Hp_I35757_IG27701_L417	61	2
88	Myoglobin	Hp_C00053_IG00001_L722	57	2
89	Lysozyme-2	Hp_I05758_IG01053_L926	56	2
90	NSP-033	Hp_I13332_IG05276_L1776	52	5
91	NSP-003.3	Hp_I01045_IG00068_L1382	52	2
92	Lysozyme-8	Hp_I18858_IG10802_L799	52	2
93	TTR-02	Hp_I07157_IG01645_L560	51	2
94	(unknown)	Hp_I09912_IG03040_L752	50	1
95	Dipeptidyl Peptidase Four (IV) family member FRAGMENT	Hp_I21073_IG13017_L680	49	1
96	Lysozyme-4	Hp_I03085_IG00332_L1160	49	2
97	NSP-032	Hp_I20274_IG12218_L717	47	2
98	MSP-domain containing protein	Hp_I10419_IG03294_L714	44	1
99	Ribonucleotide reductase family member	Hp_I06752_IG01438_L1571	44	1
100	(unknown)	Hp_I34246_IG26190_L433	43	2

101	LSN-01	Hp_I20393_IG12337_L714	42	1
102	(unknown)	Hp_I04874_IG00782_L1023	40	2
103	VAL-07.2	Hp_I01453_IG00104_L978	40	2
104	Zinc metalloprotease-3	Hp_I12518_IG04462_L2600	39	1
105	(unknown)	Hp_I14286_IG06230_L1419	39	1
106	15kDa ES antigen	GWDWRH002BYY0F	38	1
107	(unknown)	Hp_I22552_IG14496_L630	38	1
108	TIL domain containing protein	Hp_I05827_IG01076_L1116	37	1
109	NSP-003.1	Hp_I22470_IG14414_L621	37	1
110	NSP-17	Hp_I20319_IG12263_L706	37	1
111	Astacin protease family member	Hp_I02109_IG00176_L1606	37	1
112	(unknown)	Hp_I21182_IG13126_L679	36	1
113	(unknown)	Hp_I02742_IG00275_L749	36	1
114	NSP-019	Hp_I21313_IG13257_L675	36	3
115	Kunitz inhibitor	Hp_I45281_IG37225_L323	36	1
116	(unknown)	Hp_I12715_IG04659_L2277	35	1
117	HSP70	FL8UM6J01BLY7J	35	1
118	Sushi-like	Hp_I08175_IG02172_L1570	35	1
119	(unknown)	Hp_I11671_IG03920_L408	35	1
120	Aspartyl protease inhibitor	Hp_I15089_IG07033_L1228	35	1
121	Cystatin	Hp_I02126_IG00179_L738	35	1
122	(unknown)	HICN8C104ERRWE	34	1
123	(unknown)	GNK0QLK03FKIGI	34	1
124	CSN-05	Hp_I14935_IG06879_L1258	34	1
125	(unknown)	Hp_I16096_IG08040_L1070	33	1
126	(unknown)	Hp_I04541_IG00689_L698	33	1
127	(unknown)	HICN8C105GEHHW	33	1
128	VAL-17	Hp_I00825_IG00052_L2998	33	1
129	Sushi-like	Hp_I16083_IG08027_L1071	33	1
130	glyceraldehyde-3-phosphate dehydrogenase (only in egg ES)	Hp_I06838_IG01481_L1319	31	1
131	C-type lectin (only found in egg ES)	Hp_C00269_IG00001_L1007	31	1
132	ACE-1	Hp_I12803_IG04747_L2174	30	1
133	NSP-018	Hp_I10136_IG03152_L609	28	1
134	Protein disulfide isomerase	Hp_I13312_IG05256_L1794	28	1

Abbreviations: VAL – Venom Allergen-like protein; APY – apyrase; CSP – conserved secreted protein with signal peptide; NSP – novel secreted protein with signal peptide; CSN – conserved secreted protein with no signal peptide; NSN – novel secreted protein with no signal peptide; ACE – acetylcholinesterase; MSP – major sperm protein.

Table 4.4

Proteins found in both fraction 9 and fraction 17 after cross-referencing of LC-MS/MS results.

Protein	ID code	Rank in fraction 9	Rank in fraction 17
VAL-1.2	Hpb-VAL-1.2	2	26
VAL-1.3	Hp_I04202_IG00583_L1521	3	53
VAL-1.4	Hp_I08791_IG02480_L1486	8	30
VAL-1.5 FRAGMENT	Hp_I22486_IG14430_L629	7	22
VAL-1.7 FRAGMENT	Hp_I17933_IG09877_L867	10	15
VAL-2.2	Hpb-VAL-2.2	17	1
VAL-19.1	Hp_I14044_IG05988_L1484	36	70
NSP-3.1	Hp_I22470_IG14414_L621	47	109
NSP-4	Hp_I24607_IG16551_L570	11	23
NSP-6	Hp_I25440_IG17384_L551	9	42
NSP-18	Hp_I10136_IG03152_L609	20	133
Unknown (new NSP/NSN)	Hp_I14567_IG06511_L1339	41	45
Aspartyl protease inhibitor	Hp_I15089_IG07033_L1228	19	120
Chitinase	Hp_I13874_IG05818_L1547	46	7
Legumain	Hp_I02849_IG00289_L1962	22	4
Vitellogenin	Hp_I01061_IG00070_L5128	5	19
Vitellogenin	Hp_I12337_IG04281_L4997	4	56
Sushi-like domain containing protein	Hp_I16462_IG08406_L1016	29	44
Sushi-like domain containing protein	Hp_I24007_IG15951_L583	24	72
Keratin-like protein	Hp_I18857_IG10801_L798	39	20

Discussion

The ability of parasite products to divert and modulate the immune response against them is widely documented but specific mechanisms are still unclear (Maizels *et al* 2004). *H. polygyrus* produces a wide variety of molecules in its ES products (Hewitson *et al* 2011b; Moreno *et al* 2011). HES has been shown to induce regulatory cell types, including regulatory DCs, and T and B cells (Maizels *et al* 2011), and to modulate immune responses in several disease models (McSorley *et al* 2013). The ability of HES to induce FoxP3 expression in naïve T cells through TGF-β activity has been published by our laboratory (Grainger *et al* 2010), however, no protein homologues of TGF-β have been found in proteomic analysis of total HES. Here I have attempted to narrow the list of potential candidates for this activity using a variety of techniques. Firstly, fractionation of HES by two methods has concentrated TGF-β activity into two distinct peaks. Content of VALs 1-4 were measured in the fractions as these have been found to be the most abundant and immunodominant proteins in HES (Hewitson *et al* 2011a; Hewitson *et al* 2011b). Proteomic analysis of the two TGF-β positive fractions produced a short-list of 20 common proteins.

Of the 20 common proteins in fraction 9 and fraction 17, some comment can be made as to the suitability of each as a TGF-β homologue candidate. Although aspartyl protease inhibitors have been found to be highly immunogenic and abundant in several helminth parasites (Shaw *et al* 2003; De Maere *et al* 2005; Delaney *et al* 2005), they do not seem to convey any TGF-β-like properties. Chitinases are commonly found in developmental stages of helminths to assist in egg hatching and exsheathment (Arnold *et al* 1993; Geng *et al* 2002; Wu *et al* 2008), and a chitinase has been shown to augment upregulation of TGF-β-receptors and signaling in fibroblasts *in vitro*, by interacting with TGF-β (Lee *et al* 2012). Another developmental protein present is vitellogenin, a major egg yolk protein, found in the proteomic analysis of HES as highly abundant (Moreno *et al* 2011).

Legumain is an asparaginyl endopeptidase, a family of cysteine proteases. This family is found in adult helminths although their precise function is unclear (Dalton *et al* 1995). The activation of other proteases within the worm is thought to be an

important one (Dalton *et al* 2009), however literature searches unveil no link with TGF-β activity. The same is true for VALs, which may be present in these fractions as a result of being the most abundant of all proteins in HES (Hewitson *et al* 2011b). The presence of VALs in the MS results confirms the finding of VAL-1 in both fractions 9 and 17 by ELISA, and demonstrates the variation in amino acid sequence in this family, as five variants of VAL-1 are found in the list of common proteins from these fractions (Table 4.4). Confirmation that VALs 1-4 do not convey any TGF-β activity (Fig 4.7) allows focus on other proteins as potential candidates for the activity in HES.

By definition, the novel secreted proteins (NSPs) in HES have no known function or great sequence similarity to other known proteins. These could therefore be good candidates for the TGF-β activity. A protein was detected in this analysis that could not be identified from the *H. polygyrus* database. Hp_I14567_IG06511_L1339 shows no BLASTX similarities and no putative conserved domains. The presence of a signal peptide cleavage site, predicted by online software SignalP, is ambiguous, and therefore the designation of this protein as an NSP (novel secreted protein with signal peptide) or NSN (novel secreted protein without signal peptide) is undecided (Fig 4.9).

Some limitations to the methods used should be considered. The detection of a single protein responsible for the TGF-β activity in HES may be misguided, as a combination of molecules may interact to have this effect, which digestion during LC-MS/MS would mask. There may also be detection limitations in that the parameters used in the mass spectrometry may not allow detection of the protein of interest. However, considering the strikingly discrete presence of TGF-β activity in just one fraction in each of the methods of separation, there does seem to be an enrichment of this molecule, without separation of any co-factors that may have been involved in total HES. Ability of the single fractions to upregulate FoxP3 expression in naïve splenocytes could be undertaken, in an experiment similar to that showing HES itself had Treg inducing abilities (Grainger *et al* 2010). Further investigation into the common 20 molecules in fractions 9 and 17 is currently underway by

members of the laboratory, with the goal to express them as recombinant proteins, and to test each as potential immunomodulators and vaccine candidates.

As *H. polygyrus* and indeed total HES (Hartmann *et al* 2009; Liu *et al* 2009; Hang *et al* 2010; McSorley *et al* 2012), have been shown to modulate immune pathologies associated with common disease models, it is an attractive prospect that a single molecule in HES could be used in the same way, as an immunomodulator, in a therapeutic setting.

Key Findings

- The TGF-β activity in HES can be narrowed to distinct fractions in both gel filtration and anion exchange methods of fractionation, and this is repeatable and robust, giving a reliable method for continued fractionation of different batches of HES and analysis of the contents of different fractions for immunomodulatory components.
- Proteomic analysis of TGF-β-positive fractions, and cross-referencing of results, led to a shortlist of 20 common proteins to the two fractions. These included VAL proteins, which are the most immunodominant protein family in HES, but when recombinent versions of VALs 1-4 were tested on the TGF-β assay the results was negative.
- Further investigation into the NSPs could yield positive results as these, by their very nature, have no know structure or function, and could be conferring TGF-β activity to HES.

Final discussion

In order to control and eliminate macroscopic parasites, such as helminths, the host immune system must be diverse, adaptable and wide-ranging. The mechanisms involved in protection differ for each parasite and the niches inhabited by different parasites determine the array and nature of the immune response involved, as well as underlying coinfections, autoimmune diseases and tumours. Damage by migration of parasites, and immunopathology caused by over exuberant responses, must also be controlled and limited by the immune system, and these regulatory mechanisms are a facet of the host response that helminths have evolved to exploit and induce to prolong their own survival. In the face of such a complicated array of mechanisms and systems, study of single helminth infections, in controlled settings, such as inbred mouse strains, allows a close dissection of single subsets and molecules that are involved.

This thesis has attempted to study various aspects of the primary immune response, principally to *H. polygyrus*, an excellent and widely-used model of a helminth infection soley restricted to the gastrointestinal tract. Specific mechanisms involved in protection against this parasite are not fully understood, and research is ongoing into both innate and adaptive arms of the response. However, the induction of a strong Th2 response, which is vitally important in resistance (Urban *et al* 1991b; Svetić *et al* 1993), and a strong T reg response to both the worm and its ES products (Finney *et al* 2007; Rausch *et al* 2008; Grainger *et al* 2010), have been well characterised.

Firstly, in Chapter 1, the role of B cells and antibodies in the immune response to *H. polygyrus*, during a primary infection, were investigated with the use of various genetically-modified mouse strains. Convincing findings in recent literature have shown that B cells are necessary for protection after a secondary challenge infection with *H. polygyrus* (McCoy *et al* 2008; Wojciechowski *et al* 2009; Liu *et al* 2010b), although to what extent the roles of B cells such as production of cytokines, production of polyclonal or antigen-specific antibodies or antigen presentation, contribute to the response is unclear. The experiments in Chapter 1, confirmed that

the absence of B cells (μMT mice), production of antigen-specific antibody (MD4 mice) and stimulation of a full B cell response, including class switching and induction of a strong Th2 response ($CD40^{-/-}$ and $CD154^{-/-}$ mice), had only small effects on parasitological outcome after a primary infection in poorly resistant C57BL/6 mice. Collectively, these results suggest that, upon first exposure to *H. polygyrus,* cell types other than B cells are more important in protection, although the role of B cells in the formation of granulomas, which are thought to contribute to damage of migrating larvae in the intestinal wall, was complicated by the surprising finding that μMT mice cannot form granulomas. Further investigation into reasons for this would be important, as the role of the granuloma has not been fully explained, and no other studies on *H. polygyrus* in B cell-deficient mice have quantified or qualitatively described the granulomas.

Although antigen-specific antibody has been shown to be important in secondary responses (McCoy *et al* 2008), the range of molecules in HES strongly bound by antibodies shows a remarkably similar profile using primary and secondary serum on Western blots. Experiments presented here characterise mAbs that bind the immunodominant molecules in HES, previously identified as VALs, and confirm the dominance of IgG1 as the prominent antibody subclass after infection with *H. polygyrus*. Although the IgG1 antibody fraction has previously been identified as the most protective (Pritchard *et al* 1983), transfer of mAbs raised to HES tested so far have not conferred protection. The role of antibodies in protection against helminths is as yet not fully understood, and further investigation into why certain molecules are immunodominant, and how we can use them as tools to diagnose, prevent or cure helminth infections in animals and humans is of importance to basic parasitological understanding and of concern to worldwide healthcare.

A broader study of a number of cellular subsets involved in primary immunity to *H. polygyrus* is described in Chapter 2, where four strains of mice with a spectrum of parasitological outcomes were scrutinised. Aspects of immunity including strength of the Th2 cytokine response and serum IgG1 titre were confirmed here having been observed in previous publications (Prowse *et al* 1978; Wahid and Behnke 1993a; Lawrence and Pritchard 1994). Interestingly, the relatively new cell subset, ILCs,

that initiate a Th2 response, and which have not been characterised in *H. polygyrus* infection to date, were found to correlate with resistance in terms of proportion and total numbers in the MLN. These cells have been studied in several helminth infection and allergy models, and use of cytokine-reporter mice in an early time-course experiment would help to dissect further the movements, kinetics and interactions of ILCs in *H. polygyrus* infection.

Furthermore, the prominence of AAMΦ in the granuloma and gut tissue in more resistant mice was illustrated, having previously thought to be more important in a secondary infection setting (Anthony *et al* 2006; Patel *et al* 2009). Indeed, the importance of AAMΦ in granulomas was illustrated by experiments with clodronate, which significantly decreased granuloma numbers and protection in depleted mice, as did a deficiency in IL-4Rα. It will be very important in the future to dissect the exact cellular composition of granulomas and how they are involved in protection against *H. polygyrus*. Although most characterization has been on secondary infection, these experiments have shown a strong correlation between numbers of granulomas and resistance in a primary setting.

Chapter 3 focussed on the role of MIF in anti-helminth immunity. Although MIF has been well characterised in terms of promoting inflammatory diseases, tumours, and wound healing, and has pleiotropic effects in the body, its role in anti-helminth immunity is much less well characterised. Due to its link with the alternative activation of macrophages in a Th2 setting (Prieto-Lafuente *et al* 2009), $MIF^{-/-}$ mice were utilized to investigate this aspect of the immune response to *H. polygyrus* further, and additionally, the acute migratory helminth parasite *N. brasiliensis*. A dramatic and reproducible highly-susceptible phenotype was observed in these mice after infection with both parasites, although no defect in adaptive immune responses was observed, in in the formation of granulomas in *H. polygyrus* infection, suggesting that these are not solely responsible for worm killing, and that innate cellular populations are more important than T cells, B cells or regulatory processes in the protective effector mechanism.

Several aspects of innate immunity were affected by a lack of MIF, including macrophage alternative activation, eosinophilia, induction of $CD11b^{+}$ $Gr1^{+}$ cell

subsets, and induction of ILCs after infection with *H. polygyrus*, and as the subsequent Th2 response is not compromised in these mice it will be important to assess more thoroughly the impact of these defects in other areas of protective immunity. In order to do this comprehensively, an approach utilizing microarray technology on gut tissue from wild-type and MIF-deficient mice, or from individual cell types, would be necessary, to observe the impact on expression of genes and induction of pathways.

A possibility is that MIF may be acting as an alarmin, like IL-25, IL-33 and TSLP (Saenz *et al* 2008) to induce ILCs after epithelial damage by the migrating parasite. The interesting observation that in *N. brasiliensis* infection, there is no apparent defect in ILC induction indicates that there may be redundant factors induced by the worms that could be being selectively suppressed by *H. polygyrus* but not *N. brasiliensis*. This is supported by the fact that in uninfected mice, MIF-deficiency does not confer an inability to induce ILCs in response to exogenous IL-25 or IL-33, or even in the ability to make IL-33 in response to a potent airway stimulus of this cytokine. Further testing of the ability of MIF to induce ILCs *in vitro,* and the assaying of mediators released from epithelial cells upon stimulation with *H. polygyrus* or HES will be important. Indications of the presence of MIF in epithelial cells and granulomas after *H. polygyrus* infection was shown by immunohistochemical staining, but more sophisticated fluorescent microscopy could be used to further map the storage of this cytokine in different cells of the gut.

Whilst the defect in ILCs seemed to be parasite-specific, significantly lower induction of $CD11b^+$ $Ly6G^+$ and $CD11b^+$ $Ly6C^+$ cells was noted after infections with both *H. polygyrus* and *N. brasiliensis,* in $MIF^{-/-}$ mice compared to wild-type mice. These markers alone do not enable the exact identification of a specific cell type, although several researchers have used these to determine that cells were MDSCs, neutrophils, inflammatory monocytes and suppressive monocytes (Sunderkotter *et al* 2004; Zhu *et al* 2007; Brandau *et al* 2013; Pillay *et al* 2013). The work presented here is very preliminary in this area, and so further work should assess the exact characteristics of these myeloid cells, including whether they can be induced to develop from bone marrow precursors by culture with rMIF, whether they have

suppressive properties, their molecular expression and whether $MIF^{-/-}$ still fail to induce them in settings known to have high levels of MDSCs, for example in tumour models.

The successful development of a protocol to chemically inhibit MIF with 4-IPP allows research in any strain of mouse, without being restricted to the $MIF^{-/-}$ genotype. This could facilitate a wide range of experiments to assess the importance of MIF in other parasite infections for example, or to see if inhibition of MIF can overcome even the most resistant phenotype, such as SJL mice, to *H. polygyrus*. The use of this chemical allowed the demonstration that similar defects in the immune response could be recapitulated without genetic manipulation. Unfortunately, the same was not found for rMIF, which did not rescue $MIF^{-/-}$ mice in terms of parasitological outcome in *H. polygyrus* or *N. brasiliensis*, when administered i.p. Different sites of injection should be investigated in the future, as the chemoattractant properties of MIF may be affecting migration of cells to the site of infection and preventing it from working as it would in a normal infection setting. It was apparent however that rMIF induced expression of markers of alternative activation RELM-α and Ym-1, and numbers of $CD11b^{+}$ $Ly6C^{+}$ and G^{+} subsets in both the PL and in the gut tissue, although the same was not true for ILCs.

These experiments have illustrated the pleiotropic roles of MIF in immunity, several of which have not been documented before with relation to helminth infections. Therefore it will be important to investigate the finer points of how MIF is working in induction, activation or promotion of these mechanisms.

Finally, work included in Chapter 4 attempted to investigate the molecule in HES responsible for the TGF-β activity that has previously been observed to induce conversion of $Foxp3^{-}$ T cells to $FoxP3^{+}$ T regs *in vitro* (Grainger *et al* 2010). Using two methods of protein fractionation, peaks of TGF- β activity were isolated within distinct fractions, and results from LC-MS/MS of these two fractions were cross-referenced to produce a shortlist of 20 potential candidates. As several VAL proteins were identified in the list, and have previously been shown to be highly immunodominant and abundant (Hewitson *et al* 2011a; Hewitson *et al* 2011b), bacterially-expressed recombinant versions of VALs 1-4 were analysed for TGF-β

activity but resulted in no signal when compared to whole HES. The use of other methods to express VALs will be necessary to ensure correct folding and post-translational modifications, as will investigation of the other candidates on the list as to their TGF-β activity. Even more extensive fractionation may also reveal other candidates or eliminate some from the list.

These experiments are a prelude to future work assessing a variety of molecules found in HES with different properties. HES has been shown to dampen allergic airway inflammation without the need for a live worm infection (McSorley *et al* 2012), and so it will be very important to dissect the active immunomodulatory components in order to thoroughly assess how immune suppression is occurring and how HES, or its components, could be used as therapy for other disease models. This will also serve to contribute to the wider body of knowledge that could result in helminth therapy for a variety of debilitating human diseases (Elliott and Weinstock 2009; Fleming 2013; Weinstock and Elliott 2013).

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Semin Immunopathol (2012) 34:829–846
DOI 10.1007/s00281-012-0347-3

REVIEW

Immunity to the model intestinal helminth parasite *Heligmosomoides polygyrus*

Lisa A. Reynolds · Kara J. Filbey · Rick M. Maizels

Received: 15 June 2012 / Accepted: 13 September 2012 / Published online: 11 October 2012

Abstract *Heligmosomoides polygyrus* is a natural intestinal parasite of mice, which offers an excellent model of the immunology of gastrointestinal helminth infections of humans and livestock. It is able to establish long-term chronic infections in many strains of mice, exerting potent immunomodulatory effects that dampen both protective immunity and bystander reactions to allergens and autoantigens. Immunity to the parasite develops naturally in some mouse strains and can be induced in others through immunization; while the mechanisms of protective immunity are not yet fully defined, both antibodies and a host cellular component are required, with strongest evidence for a role of alternatively activated macrophages. We discuss the balance between resistance and susceptibility in this model system and highlight new themes in innate and adaptive immunity, immunomodulation, and regulation of responsiveness in helminth infection.

L. A. Reynolds and K. J. Filbey contributed equally to this manuscript.

This article is a contribution to the special issue on Immunoparasitology—Guest Editor: Miguel Stadecker

This article is published as part of the Special Issue on Immunoparasitology [35:1]

L. A. Reynolds · K. J. Filbey · R. M. Maizels
Institute of Immunology and Infection Research,
University of Edinburgh,
Edinburgh EH9 3JT, UK

R. M. Maizels (✉)
Institute of Immunology and Infection Research,
University of Edinburgh,
West Mains Road,
Edinburgh EH9 3JT, UK
e-mail: rick.maizels@ed.ac.uk

Introduction

Heligmosomoides polygyrus: a model organism

Chronic helminth infections remain a huge global health problem, causing extensive morbidity in both humans and livestock. Many of the most prevalent helminth parasites are difficult to study in the laboratory, as they have co-evolved with, and are closely adapted to, their definitive host species. However, model organisms such as *Heligmosomoides polygyrus*, a natural mouse parasite, offer tractable and informative systems to explore the mechanisms of immunity and immune evasion in helminth infections [1, 2].

H. polygyrus (previously named *Nematospiroides dubius*) is an intestinal nematode parasite in wild mouse populations that has successfully been transferred to the laboratory. It is phylogenetically placed in the same Suborder, Trichostrongylina, as the ruminant parasites *Haemonchus contortus* and *Teladorsagia circumcincta* and within the same Order, Strongylida, as the human hookworm parasites *Ancylostoma duodenale* and *Necator americanus* [3]. *H. polygyrus* is an appropriate model of these chronic helminthiases, as primary infections can persist for many months in susceptible strains of mice.

In an experimental setting, *H. polygyrus* is introduced by orally gavaging mice with infective L3 larvae. Following ingestion, within 24 h, larvae have penetrated through into the submucosa of the small intestine. Here they undergo two developmental molts, before emerging back into the lumen as adult worms, which feed on host intestinal tissue [4]. The adult worms coil around the small intestine villi to secure themselves, mate, and produce eggs, which are excreted in the feces. In the external environment, the eggs hatch and undergo two molts to become infective L3s, and so the lifecycle continues (Fig. 1).

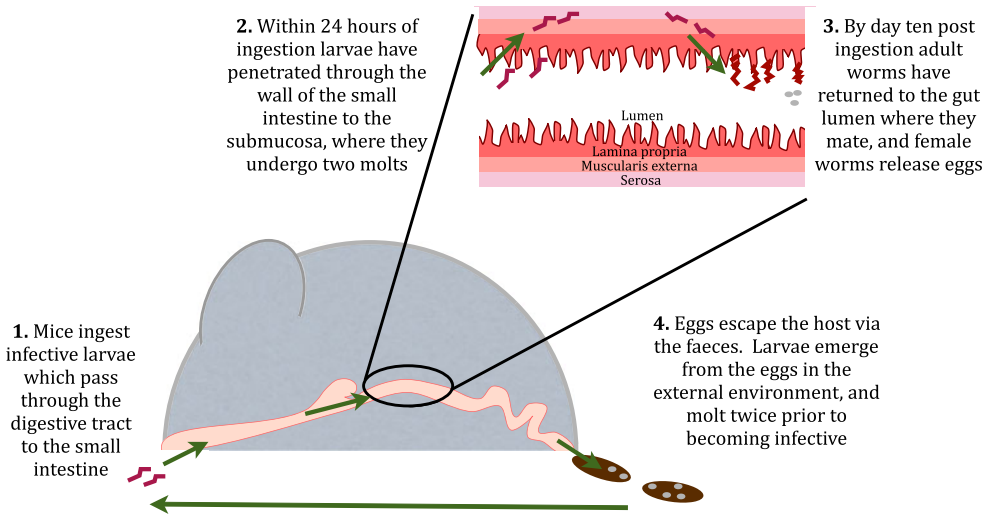

Fig. 1 Lifecycle of *Heligmosomoides polygyrus* in mice

The persistence of *H. polygyrus* within the murine host can be measured by determining the number of eggs that are released in the feces, or by enumerating adult worms remaining in the small intestine. As described below, the wide range of reagents available for assaying and manipulating the murine immune system cells in mice are being effectively applied to investigate responsiveness and immunity. The mechanisms behind helminth expulsion in mice can therefore be studied in order to make predictions about similar interactions between helminths and the immune system in livestock and humans, with a view to developing much-needed vaccines for control of these infections.

A further advantage of *H. polygyrus* is that the mammalian stages can be cultivated in vitro, where its secretory products, *H. polygyrus* excretory–secretory antigens (HES), can be collected, and individual components can be purified and identified [5]. This provides a fruitful strategy to test defined parasite molecules in vitro and in vivo for immunomodulatory functions and as candidate vaccine antigens.

Genetics of susceptibility to *H. polygyrus*

In primary infections of different mouse strains, the length of time *H. polygyrus* can persist and the degree of response it provokes shows considerable variation, and some genotypes are also poor at rejecting challenge infections following immunisation.

Table 1 shows a summary of "responsiveness" to *H. polygyrus* in different mouse strains, based on adult worm survival and fecundity after primary and secondary infection. The genetic factors controlling strain differences in resistance to infection include the major histocompatibility complex (MHC) H-2 loci, with weak responders among the H-2^k and H-2^b genotypes and the H-2^q or H-2^s genotypes associated with a rapid response [6, 7].

Experiments in H-2 congenic C57BL/10 mouse strains show that although establishment of *H. polygyrus* larvae is equal between all strains (shown by worm counts 2 weeks postinfection), by week 9, egg and adult worm numbers differ strikingly. Those with H-2^s and H-2^q haplotypes expelled the parasites more rapidly [7, 8], while mice carrying H-2^b or H-2^k haplotypes backcrossed into the fast-responding BALB/c background were unable to expel worms quickly [7]. Resistance was shown to be conferred by more than one gene, as F1 hybrids of fast responders, SJL and SWR, display heightened abilities to expel worms, and is inherited in a dominant fashion as C57BL/10xSJL hybrids are as rapid in expulsion as the SJL parental strain [7, 9].

More recently, a study mapping quantitative trait loci in fast responding (SWR, H-2^q) versus slow responding (CBA, H-2^k) strains found significant effects on resistance to *H. polygyrus* trickle infection from positions on chromosomes 1, 2, 13, and 17 [10]. Several candidate resistance genes were identified, including as expected MHC (on chromosome 17), and also interleukin-9 (IL-9; on chromosome 13), both of which correlate with worm expulsion [10].

A notable gender bias in susceptibility is also observed, with female mice of all strains clearing primary infections faster than their male counterparts as also apparent to a lesser degree following secondary exposure [11–13]. The greater susceptibility of male mice correlates with higher fecundity of worms recovered from male hosts and a larger adult worm body size [14], indicating that the parasites are fitter than those from a female host.

Concurrent pregnancy and worm infection imposes increased physiological demands on the mother in terms of the energy required to fight infection and to nourish the fetus. This can lead to immunosuppression (diminished Th2 responses) [15] and adverse reproductive outcomes (small pup size) [15, 16]. The effects of pregnancy on maternal serum cytokines during *H. polygyrus* infection include

Table 1 Strain-specific immunity to *H. polygyrus*

Responsiveness	Strain	Immune mechanisms investigated
Slow (>20 weeks to expel worms)	CBA C3H SL A/J	Primary response involves significantly lower cell numbers in the MLN than other strains [193, 194], very few mast cells in the gut [193], low levels of mMCP in serum and intestinal lavage [51, 53], and low eosinophilia [14] Have no, or very weak, protective response to re-challenge [6, 53, 195]
Intermediate (8–20 weeks)	C57BL/6 C57BL/10 129/J	C57BL/10 mice show less rapid and lower eosinophilia levels in circulation, after both primary *H. polygyrus* infection or injection of parasite antigens, than NIH mice [196]
Fast (6–8 weeks)	DBA/2 BALB/c NIH	NIH mice produced a higher peak of lymphocytosis, neutrophilia and monocytosis in the circulation than C57BL/10 mice after primary infection [197]
Rapid (4–6 weeks)	SJL	SJL and SWR have quicker and stronger antibody responses than other strains, involving stronger recognition of a larger number of antigens on a Western blot of HES [198] and adult homogenate [193], and higher titers of parasite-specific antibody of different isotypes in serum [51, 193, 198]
	SWR	Infected SWR MLN cells produced higher levels of IL-3, IL-4 and IL-9 after ConA stimulation than NIH and CBA [52] Both strains show early peaks of serum tumor necrosis factor alpha, mMCP-1, intestinal mast cells and goblet cells, which precede the expulsion of the worms [51, 53]

increased levels of IL-1β and IL-6 at day 20 postinfection [15] and lower concentrations of IL-4, IL-5, IL-13, and mucosal mast cell protease (mMCP-1) [15]. Pregnant mice also show a small but significant increase in adult worm burdens [15].

Models of resistance

Immunity to *H. polygyrus* can be studied in three separate settings each with distinct implications for human infection, namely, genetically determined, drug-induced, and vaccine-elicited immunity. In each of these contexts, the availability of numerous gene-targeted mouse strains and immunological reagents are being used to define immune system components and parameters required for immunity to infection.

As stated above, the outcome of primary *H. polygyrus* infection is strongly influenced by the genetic background of mice, with strains differing in their susceptibility to chronic infection. Studying how the immune response differs between those strains that endure chronic infections and those that are able to clear a primary infection has been highly instructive in defining the immune mechanisms the host must promote in order to clear the parasite.

If primary infection with *H. polygyrus* is cleared using antihelmintic drugs such as pyrantel pamoate or ivermectin [17], most mouse strains display a highly effective memory response, which provides immunity to reinfection [18]. Genetic background also impacts on resistance to reinfection, as BALB/c mice display significantly lower worm numbers postchallenge compared to C57BL/6 [19, 20].

Finally, HES administered in alum adjuvant has been shown to induce sterilizing immunity to *H. polygyrus* infection [21], and studies are ongoing to identify the specific components of HES and the immune mechanisms critical for this immunity.

Challenge infection/Trickle infection

Although most laboratory studies employ a single bolus infection, doses used are far from physiological or representative of field conditions. Hence, some investigators have developed trickle infection regimes, for example, administering twice weekly low doses of infective larvae. Under these conditions, different mouse strains show a gradation of resistance patterns similar to those seen with single-bolus primary infection, in that NIH and SWR strains resolve infection (showing an initial increase in adult worm burden, a period of stability, and then finally expulsion) while CBA and C57BL/10 mice continue to accumulate increasing adult worm burdens over the course of repeated infections [22].

Variation and adaptation by *H. polygyrus*

The strain of *H. polygyrus* used in laboratories worldwide is thought to have been isolated from wild Californian mice in the 1940s [23] and was known for some years as *Nematospiroides dubius*. The vast majority of the literature describing

experiments with this isolate refers to the parasite as *H. polygyrus*. It was, however, suggested that this laboratory strain should be referred to as *H. polygyrus bakeri*, to differentiate it from wild strains of the parasite, considered to be *H. polygyrus polygyrus* (found in the wood mouse *Apodemus sylvaticus* in Europe), *H. polygyrus corsicus* (from the house mouse *Mus musculus* in Corsica) and *H. polygyrus americanus* (from the vole *Phenacomys intermedius* in North America) [24]. More recently, there has been an additional proposal of a name change for the laboratory isolate to *H. bakeri* [23], based on sequence divergence between laboratory and European wood mouse isolates [25]. This proposal has not received widespread support due to the preliminary nature of the data, the sequence variation even within the laboratory strain, and the need to remain consistent with previous literature [26]. Here, we refer to the laboratory strain of the parasite as *H. polygyrus*.

In proteomic studies on *H. polygyrus* secreted antigens (see below), extensive sequence variation was observed in some gene families [5], indicating that despite many years of laboratory propagation, the parasite strain remains highly polymorphic. Moreover, there are indications that antigen expression by *H. polygyrus* may vary or adapt according to the host strain of mouse [27, 28], with proteomic differences in adult worms recovered 4 weeks postinfection in either C57BL/10 (slow responder) or SWR (fast responder) hosts [28]. Phosphatidylethanolamine-binding protein and several nematode globins are overexpressed in worms from C57BL/10 compared to worms from SWR mice, and myosin, troponin, actin, and several unidentified proteins are overexpressed in the worms from fast responder mice compared to slow [28]. Differential expression of worm products in different host strains may shed light on pathways targeted by the immune system that impact on worm survival or death.

Host immune responses

The critical requirement for the adaptive immune response in control of the parasite is illustrated when B- and T-cell responses are lacking. Severe combined immunodeficient (SCID; B- and T-cell deficient) and athymic mice show impaired expulsion of adult worms, maintaining high worm burdens several weeks post infection by which stage wild-type counterparts had expelled the majority of their worms [29, 30]. Treatment with anti-CD4 results in higher fecundity of female worms in a primary infection [31] and transfer of the effector T-cell subset from chronically infected animals significantly reduced worm burdens when transferred to naive mice before infection [32]. Although B-cell deficiency does not affect the outcome of primary *H. polygyrus* infection, B-cell or antibody deficiency significantly compromises the ability to expel a secondary challenge infection [33].

T-cell responses

H. polygyrus infection induces a strongly polarized Th2 response, which has been shown to be critical in control and expulsion of the worm [31]. A primary *H. polygyrus* infection induces IL-3, IL-4, IL-5, and IL-9 gene expression in the mesenteric lymph nodes (MLN) and Peyer's patches [34] and elicits the release of high concentrations of IL-4, IL-5, IL-9, IL-10, and IL-13 protein from MLN, spleen, and lamina propria mononuclear cells (LPMC) cultured with parasite antigens [32, 35, 36]. IL-4 is the most critical single cytokine for protection against primary and secondary *H. polygyrus* infection (both in expulsion of adult worms and inhibiting their egg production) [31]. Immunity to secondary infection is diminished by blocking antibody to IL-4 but completely abolished when the IL-4R is also blocked [31]. This suggests that IL-13, which also signals through IL-4Rα, can partially compensate for the loss of IL-4, but in the absence of signaling from both cytokines, protection against reinfection with *H. polygyrus* is lost. Blocking of IL-5 by antibody treatment had no effect on worm expulsion [31]. When IL-4 is administered as a complex with anti-IL-4 (IL-4C) to extend the activity time of this cytokine, wild-type BALB/c mice expelled *H. polygyrus* more rapidly [29]. This effect did not depend on the adaptive immune system, as *H. polygyrus* expulsion was also seen in SCID mice and anti-CD4-treated BALB/c mice, which were given the IL-4 complex [29]. After primary infection, $CD4^{+}IL\text{-}4^{+}$ T cells disseminate around the body to lymphoid and nonlymphoid organs, such as airways, peritoneal cavity, and liver, and have a lower apoptotic potential [37, 38]. These findings may be illustrative of peripheral reservoirs of long-lived memory Th2 cells primed to respond to subsequent infection challenges by the worm.

Further studies have delineated the costimulatory signals required to mount Th2 responses to *H. polygyrus* infection. By blocking signaling through both CD80 (B7-1) and CD86 (B7-2) with specific antibodies or a CTLA4-Ig construct, IL-4 expression, Th2 expansion, and IgE production in response to *H. polygyrus* were ablated [39, 40]; interestingly, an innate IL-5 response remained intact when T-cell costimulation was inhibited. Blocking antibodies against CD80 or CD86 alone had little effect [40], and while early (day 6 postinfection) responses to *H. polygyrus* were unaltered in CD86-deficient mice [41], by day 14 postinfection, CD86-deficient mice had higher parasite egg burdens and decreased Th2 responses [41]. This showed that, while CD86 was not required for the initiation of the antiworm response, it was necessary for its progression and persistence.

Although both CD80 and CD86 normally ligate to T-cell CD28, CD28-deficient mice were found to have no impairment in the early $CD4^{+}IL\text{-}4^{+}$ response, indicating an alternative mechanism for Th2 costimulation in *H. polygyrus*

infection [42]. Moreover, while the primary T-cell response to infection is CD80/CD86-dependent as outlined above, on secondary infection, memory helper T cells do not require CD80 or CD86 costimulation for their activation to protect against challenge [43, 44]. Studies into an additional costimulatory molecule, OX40L (CD143), showed that it is specifically required to promote IL-4 production from T cells (and the associated rise in IgE), without affecting Th2-cell expansion, migration, germinal center formation, or IgG1 levels [45].

T-follicular helper cells are now recognized as the instrumental subset, which induces germinal center formation and isotype switching in B cells, by migrating to B-cell follicles and releasing cytokines including IL-21 [46], while also producing IL-4 in the MLN of *H. polygyrus*-infected mice [47]. IL-21 plays key roles by stimulating multiple cell types across a range of infections [48]. In *H. polygyrus* infection, IL-21 deficiency results in reduced intestinal granuloma formation, impaired T-cell expansion and survival, and lower numbers of circulating basophils and eosinophils [49]. IL-21 also provides a critical signal for the differentiation of B cells into plasma cells and for protection against secondary challenge infection with *H. polygyrus* [50].

B-cell and humoral responses

In general, the intensity and speed of parasite-specific antibody responses are greater in more resistant mouse strains such as SJL and SWR than in susceptible strains. Specifically, the IgG1 and IgE responses (to adult worm homogenate and HES) negatively correlate across strains with worm survival after a primary infection [12, 51, 52]. However, after repeated low-dose ("trickle") infections, there was little difference between slow and fast responder strains in any antibody isotype measured to larval and worm antigens [53].

B cells, as well as secreting antibodies, also produce cytokines and costimulatory molecules that promote and amplify the T-cell response in a selective manner [54, 55]. Interestingly, the greatest increase in cell number in MLN after *H. polygyrus* infection is in the B-cell compartment [32, 56].

The protective response to secondary challenge with *H. polygyrus* is dependent on B cells, as μMT and JHD mice (both of which lack B cells) cannot clear the parasites [57–59]. Defective immunity in B-cell-deficient mice is not due to an impairment of Th2 responses, or to T regulatory cell (Treg) activation, development, or differentiation, as a pronounced local Th2 response in the intestinal tissues occurred with or without B cells, in both primary and secondary infection [58, 59]. However, a separate study showed impairment of the Th2 response in B-cell deficient mice, with significantly lower T-cell expansion and cytokine production [57]. These authors showed that a sufficient T-cell memory response was B-cell dependent and that immunity required B cells to produce the cytokines IL-2 and tumor necrosis factor alpha [57]. This discrepancy remains unclear, and B cells seem to have differing roles in other helminth infections. During a primary infection with the colon-residing murine nematode parasite *Trichuris muris*, B cells are required for resistance and the development of a Th2 response [60]; however, in primary and secondary infection with another gastrointestinal nematode, *Nippostrongylus brasiliensis*, Th2 responses and worm expulsion are B-cell independent [58].

The specific role of antibody in mediating protection against *H. polygyrus* has also been investigated using mice with targeted deficiencies within the B-cell compartment. For example, μs mice have secretory IgM-deficient B cells, but can produce parasite-specific class-switched IgG1 and IgE, and are able to clear secondary infection [57]. However, when crossed with a null activation-induced deaminase transgene, the resultant mice are unable to undergo affinity maturation or secrete antibody of any isotype and are not protected from secondary challenge with the parasite [57]. Using selective isotype knockout mice given a secondary *H. polygyrus* infection, it was found that IgE had no role in protection, and IgA had a minor role, leaving IgG as the major class-switched isotype leading to protection [59]. Indeed, it has been long known that the humoral response is dominated by parasite-specific IgG1 and that serum fractions with highest parasite-specific IgG1 activity afford greater protection when transferred to an infected animal [61]. Even transfer of whole serum from immune wild-type donors to JHD-recipient mice can significantly reduce the number of adult worms left in the intestine after a challenge infection [58].

Primary *H. polygyrus* infection also elicits an extraordinary increase in nonspecific serum IgG1 levels (hypergammaglobulinemia) [59, 61, 62], and following repeated trickle infections over 4 weeks, serum IgG1 concentrations can reach 30 times the normal level seen in uninfected mice [62, 63]. Despite these high concentrations, transfer of serum from 28-day infected mice does not protect naive animals from infection [59, 64]. In contrast, serum raised after multiple *H. polygyrus* infections is protective against adult worm survival when transferred into naive recipients [64, 65], presumably reflecting the higher ratio of parasite-specific to nonspecific IgG following repeated infection. The mechanisms through which such high levels of polyclonal IgG1 are produced in response to *H. polygyrus* (and other parasitic worms [63]) remain to be explored.

Innate immune responses

Innate immune cells are the initial responders to a *H. polygyrus* infection and are also implicated in the end-stage expulsion of parasites. Innate cells release type 2 cytokines

that can act directly to alter gut physiology and polarize the adaptive immune response, while themselves employing helminth-damaging or killing mechanisms [66, 67].

Dendritic cells

Dendritic cells (DCs) are the predominant innate antigen-presenting cell that are required to prime Th2 responses against helminths [68]. DCs loaded with helminth products in vitro can be transferred to naive animals to induce a Th2 response [69] and have been shown to inhibit allergic airway inflammation when transferred from a helminth-infected animal, resulting in increased numbers of Tregs and a downregulation of Th2-mediated inflammation [70]. When $CD11c^+$ DCs are depleted using CD11c.DTR mice [71] that coexpress CD11c with the human diphtheria toxin receptor (DTR), a Th2 response against several helminths (including *H. polygyrus*) is severely compromised [72, 73].

Macrophages

The alternative activation of macrophages is a hallmark of helminth-elicited Th2 responses and is associated with high expression of a characteristic set of gene products, including Ym1, RELM-α (FIZZ-1), arginase-1, IL-4Rα, and the mannose receptor CD206 [66, 74]. Macrophages can differentially express the enzymes nitric oxide synthase 2 (NOS-2) and arginase-1, which compete for the common substrate L-arginine, and are competitively induced by interferon gamma (IFN-γ) and Th2 cytokines (IL-4, IL-10, IL-13, and IL-21), respectively [75–78]. The activation state of macrophages in helminth infections is sufficiently plastic to respond to changing stimuli, as helminth-induced alternatively activated macrophages restimulated ex vivo with lipopolysaccharide and IFN-γ switch to a classically activated phenotype [79], suggesting that such plasticity may also occur in vivo.

Alternatively activated macrophages are critical to the protective immune response to secondary *H. polygyrus* infection, as mice lost the ability to reject challenge infections when depleted of macrophages via clodronate treatment, or when treated with *S*-(2-boronoethyl)-l-cysteine (BEC), a pharmaceutical inhibitor of arginase [80]. Arginase-1 may directly harm parasites, as *H. polygyrus* exhibited higher levels of cytochrome oxidase, a marker of a stress response, in a secondary infection compared to a primary infection, and this increase was lost following BEC administration [80]. In contrast to arginase, no antiparasite function has been found for Ym1, a member of the chitinase-like family of proteins that lacks demonstrable chitinase activity [81]. Ym1 does bind heparin on cell surfaces and in the extracellular matrix [81], which may indicate a role for Ym1 and alternatively activated macrophages in mediating repair of tissue damage caused by *H. polygyrus* when migrating through the intestinal wall [82].

Alternatively activated macrophages may also be important mediators of the smooth muscle hypercontractility response to intestinal helminth infections, at least in the context of a *N. brasiliensis* infection, as depleting macrophages via clodronate-treatment blocked smooth muscle hyperactivity and impaired worm expulsion [83].

Neutrophils

Perhaps surprisingly, the role of the principal granulocyte cell types (neutrophils, eosinophils, and basophils) has not been directly evaluated in *H. polygyrus* infection. Neutrophils are prominent in primary and, to a lesser extent, secondary granulomas during a *H. polygyrus* infection [80, 84, 85]. The finding that neutrophils are less prevalent in a setting of heightened resistance may indicate that they are not a key cell type in immunity. To date, a protective function for neutrophils during helminth infections has only been reported for infections of mice with tissue-migrating larvae of the human nematode parasite *Strongyloides stercoralis*, although even in this case killing was more effective when eosinophils were present alongside neutrophils [86, 87].

Eosinophils

No role for eosinophils in *H. polygyrus* expulsion has yet been described. In a genetic model of eosinophil deficiency, in which an eosinophil-specific site in the GATA-1 promoter is deleted [88], mice showed impaired resistance to challenge infections with *N. brasiliensis* [89]; significantly, in the absence of eosinophils, greater numbers of tissue larvae migrated to the lung, but expulsion of those parasites that subsequently reached the gut was unimpaired in the eosinophil-deficient mice. Eosinophilia in response to *N. brasiliensis* infection was blocked when mice were administered anti-IL-5 antibody [90, 91], but this had no impact on adult worm recovery [90], providing additional evidence that eosinophils are not a critical mediator of expulsion in this system. Anti-IL-5 treatment during *H. polygyrus* infection also had no impact on worm burden [92], and eosinophils within the gut wall have been reported to be inhibited during *H. polygyrus* infection in a manner reversible with anti-transforming growth factor beta (anti-TGF-β) antibody treatment [93].

Basophils

As with the other granulocytes, few studies have investigated the role of basophils in *H. polygyrus* infections. In other gastrointestinal nematode infections, basophilia is conspicuous, and their presence may be required for optimal *N.*

brasiliensis expulsion [94]. Worm expulsion of *T. muris* was impaired when basophil numbers were depleted using MAR-1 antibody [95]; however, this antibody targets the FcεRI, which is also expressed by mast cells, so this does not conclusively prove a role for basophils alone.

Mast cells

Mast cells are major players in the intestinal immune response to infection with *H. polygyrus*, as expulsion correlates with epithelial mastocytosis [52, 96] and elevated intestinal fluid levels of mMCP-1 in different murine strains [51]. Mast cells may promote helminth damage by increasing the permeability of the gut via mMCP-1-mediated breakdown of epithelial tight junction proteins [97, 98], thereby increasing luminal flow and disrupting the niche of parasitic helminths. Increased permeability of the gut in response to *Trichinella spiralis* is blocked in mMCP-1-deficient mice, which were less effective at clearing the worms than wild-type counterparts [98]. The mast cell response to *T. spiralis* (and *N. brasiliensis*) infection, however, is ablated in mice carrying an *H. polygyrus* coinfection [99] arguing that the latter parasite is able to suppress host mastocytosis to a significant degree.

Most in vivo studies on mast cells in helminth infection have involved the mast-cell-deficient mice $Kit^{W}/Kit^{W\text{-}v}$ which carry a mutated gene encoding the tyrosine kinase receptor c-kit. During *H. polygyrus* infections, these mice produce higher egg numbers than wild-type controls, indicative of impaired immunity [100]. Consistent with this, reduced egg production was seen in Tg2Rbeta mice [101], which exhibit mastocytosis.

In terms of protective immunity to adult worms, however, $Kit^{W}/Kit^{W\text{-}v}$ mice were found to be similar to wild type in slowly expelling primary *H. polygyrus* infection between 4 and 9 weeks of infection [100]. However, a more recent report has that shown $Kit^{W}/Kit^{W\text{-}v}$ mice and another mast cell deficient strain, $Kit^{W\text{-}sh}$ mice, do have impairments in *H. polygyrus* expulsion, as both strains had higher worm burdens than wild-type mice after 3 weeks of a primary *H. polygyrus* infection [102]. The same authors also showed that $Kit^{W}/Kit^{W\text{-}v}$ mice were not resistant to a secondary *H. polygyrus* infection, yet control wild-type mice were able to clear the infection [102]. The reason for the discrepancy between the reports on the ability of $Kit^{W}/Kit^{W\text{-}v}$ mice to clear *H. polygyrus* is not clear, and more studies are required to confirm the importance of mast cells during infections. If mast cells do contribute to expulsion of *H. polygyrus*, it could be via their contributions towards priming a Th2 response early in infection, as well as their potential role as a later effector cell. $Kit^{W}/Kit^{W\text{-}v}$ MLN cells did not show the high levels of *H. polygyrus*-antigen-specific Th2 cytokines produced by wild-type MLN cells in response to *H. polygyrus* [102].

It should be noted that both $Kit^{W}/Kit^{W\text{-}v}$ and $Kit^{W\text{-}sh}$ mice have defects that extend beyond a mast cell deficiency [103]. Many of the recently described subsets of lineage negative innate type 2 cells, discussed below, express c-kit, and so it is likely that some of the deficiencies of $Kit^{W}/Kit^{W\text{-}v}$ and $Kit^{W\text{-}sh}$ mice can be explained by the additional disruption of these cell types.

Innate lymphoid cells

Recently, a number of studies have identified a population of lineage marker negative innate lymphoid cells (ILC), which produce type 2 cytokines (particularly IL-5 and IL-13) in response to epithelial cell-derived cytokines, including IL-25, IL-33, and thymic stromal lymphopoietin (reviewed in [104]). IL-25 may also be derived from other cell types, such as mast cells [105], but the importance of IL-25 from this source is as yet unknown. Epithelial cells produce elevated levels of these cytokines in response to damage, thereby raising the first alarm leading to Th2 responses (reviewed in [106]). Trefoil factor 2 (TFF2) is a molecule involved in epithelial cell repair, which induces IL-33 production by epithelial cells in response to damage caused by *N. brasiliensis* [107]. $TFF2^{-/-}$ mice did not show the elevated epithelial IL-33 levels in response to *N. brasiliensis* that wild-type mice did, instead having lower serum IL-4 levels after 7 days of infection, and delayed worm expulsion [107]. Similarly, Th2 cytokine production is delayed, and *N. brasiliensis* expulsion is impaired in IL-$25^{-/-}$ mice, which correlates with the absence of a non-B non-T-cell c-kit^{+} IL-4, IL-5, and IL-13 producing population induced in infected wild-type mice or mice administered rIL-25 or rIL-33 [108–110]. A role for ILCs has not yet been reported during a *H. polygyrus* infection though it seems likely that these cell types are important inducers of Th2 responses during all intestinal helminth infections.

Gut physiology and intestinal epithelial cell function

IL-4 and IL-13, derived from innate or adaptive sources, are likely to have direct effects on the physiology of the gut as well as on effector cells that promote helminth expulsion. Although few changes in epithelial cell function are noted during primary *H. polygyrus* infection, in secondary infections, increased mucosal permeability, decreased ion absorption, and increased prosecretory effects in response to prostaglandin E2 and histamine were seen [111, 112]. Moreover, these changes were dependent on the IL-4R and STAT6 and were reproduced by IL-4C administration [111, 112]. These alterations to the worm's environment may interfere with its abilities to feed on the intestinal tissue [4] or remain wrapped around the villi in the small intestine.

Goblet cell function

Within the intestinal epithelial layer are specialized goblet cells that secrete innate defence proteins as well as large quantities of mucins, the key components of mucus. Goblet cell hyperplasia develops in response to intestinal helminth infections, including *H. polygyrus*, where hyperplasia is dependent on a functional T-cell response [30].

Enhanced mucus production has been suggested to act against helminth establishment, and it may be that specific components within the mucus play a role in control of helminth expulsion. RELM-β (FIZZ-2) is a cysteine-rich mediator expressed by goblet cells in response to IL-13 and is important for the normal control of epithelial cell barrier permeability [113, 114]. RELM-$\beta^{-/-}$ mice do not expel a secondary *H. polygyrus* infection as rapidly as wild-type mice and adult *H. polygyrus* worms treated in vitro with recombinant RELM-β prior to transfer to a new host survived less well than untreated adult worms [115]. This suggests that RELM-β is an important factor in inhibiting worm survival, perhaps by interfering with worm chemotaxis and nutrition [113, 115]. Secretion of MUC2, a major component of mucus in both the small and large intestine, is also upregulated during a *H. polygyrus* infection [30]. No evidence for a role for MUC2 in expulsion of *H. polygyrus* has yet been reported; however, MUC2 production correlates with the expulsion of *T. muris* [116, 117] and *N. brasiliensis* [118].

Smooth muscle contraction

Both IL-4 and IL-13 enhance smooth muscle contractility in the small intestine [119], a mechanism that has been shown to be important for resistance to other helminth infections including *Schistosoma mansoni* [120], *T. spiralis* [121, 122], and *N. brasiliensis* [123]. Increased intestinal smooth muscle contractility has been shown after infection with *H. polygyrus* [124]. Both *N. brasiliensis* and *H. polygyrus* infections cause an upregulation of protease-activated receptor $(PAR)_2$ messenger RNA in the small intestine, and a PAR_2 agonist caused smooth muscle contractility, which was enhanced in both parasite-infected groups and, for *N. brasiliensis* at least, was dependent on STAT6 [124]. The infection-induced hypercontractility in the presence of PAR_2 agonist was lost when nerve conduction was blocked using the neurotoxin TXX [124]. Whether smooth muscle hypercontractility plays a critical role in *H. polygyrus* expulsion has yet to be determined.

Granuloma formation

A striking phenomenon in infection is the formation of granulomas around the site of larval invasion in the intestinal tract, and they are more numerous in resistant strains of mice [125], particularly following secondary *H. polygyrus* infection. While granuloma formation is Th2 dependent, their function has yet to be determined, either in damaging larval worms encysted in the submucosal layer of the small intestine or in tissue repair after *H. polygyrus* has departed into the lumen of the gut [126]. Granulomas in both primary and secondary infection consist of neutrophils, macrophages, dendritic cells, and eosinophils; in secondary infection, $CD4^+$ Th2 cells and a high proportion of alternatively activated macrophages rapidly migrate to the site of infection to surround the larvae [80, 84, 85].

Immuno-regulatory cells in chronic infection

Regulatory T cells

Several categories of T cells exert suppressive or immunomodulatory effects, most prominently the subset of Tregs expressing the transcription factor Foxp3. Sustained expression of Foxp3 is required to maintain Treg suppressive function, as in its absence Tregs acquire effector T-cell functions [127], and conversely, the forced expression of Foxp3 confers suppressor function to $CD4^+CD25^-$ T cells [128]. Tregs are essential during infection to protect against immune-mediated pathology while still allowing a sufficiently robust response to clear the pathogen [129]. Indeed, when $Foxp3^+$ T cells are removed at early stages of an infection with *H. polygyrus*, pathology of the small intestine is significantly worse, with higher numbers of effector T cells, IL-4, and IL-13 [130].

Foxp3 is constitutively expressed in a subset of regulatory cells termed natural Tregs, but expression can also be induced in resting $Foxp3^-$ peripheral T cells. Natural Tregs develop in the thymus to limit autoreactive T cells, while inducible Tregs leave the thymus as conventional T cells and are converted through TGF-β, IL-10, and retinoic acid stimulation [129]. Treg induction is particularly favored in the intestine and gut-associated lymphoid tissues, where *H. polygyrus* resides and where TGF-β is highly enriched [32, 131]. Tregs, which express the integrin CD103 ($CD4^+CD25^+CD103^+$), are more suppressive of $CD4^+$ effector cells in vitro and release significantly more IL-10 into culture supernatants after stimulation with *H. polygyrus* primed dendritic cells than $CD4^+CD25^+CD103^-$ Tregs [32].

A strong Treg response develops in the MLN and spleen of *H. polygyrus*-infected mice, peaking at day 28 postinfection [35]. $CD25^+CD103^+$ cells are the subset in the $CD4^+$ compartment that shows the greatest increase in cell number (compared to $CD25^-CD103^-$ effector cells and $CD25^+CD103^-$ cells) [32, 35]. Most significantly, Foxp3 can also be induced in naive T cells by HES in vitro, in a manner analogous to TGF-β, due to

parasite-derived TGF-β-like activity [132] (discussed below). Inhibition of TGF-β signaling during *H. polygyrus* infection using the inhibitor SB431542 reduces adult worm burden and results in an increased Th2 response [132], while administration of anti-TGF-β neutralizing antibody has also been reported to result in lower worm numbers [93]. When TGF-β signaling is lost only on $CD4^+$ T cells, in TGF-βRII DN mice [133], there was no reduction in adult *H. polygyrus* burden compared to wild-type mice; in fact, *H. polygyrus* is more fecund [134, 135]. This is likely due to excessive IFN-γ production in the absence of $CD4^+$ TGF-β signaling, as when IFN-γ-deficient TGF-βRII DN mice were infected with *H. polygyrus*, fewer adult worms survived after 28 days than in IFN-γ-sufficient TGF-βRII DN mice [134], illustrating the importance of both TGF-β and IFN-γ in determining susceptibility to *H. polygyrus*.

H. polygyrus infection also induces $CD8^+$ Tregs in the lamina propria of the small intestine, which can inhibit T-cell proliferation in vitro in an IL-10 and TGF-β independent manner [36, 136].

Regulatory B cells

In addition to Tregs, regulatory B cells (Bregs) have also been described that produce IL-10 and TGF-β, and can dampen potentially harmful immune responses [137]. Bregs induced during helminth infections can not only downregulate pathology elicited by schistosome eggs [138] but also ameliorate immunopathologies such as multiple sclerosis [139] and anaphylaxis [140] in humans and mice. While the role of Bregs in parasite persistence has not been directly investigated in *H. polygyrus* infection, suppressive B cells expand in the MLN of infected C57BL/6 mice, which on transfer to uninfected hosts, suppress airway allergy and inflammation in experimental autoimmune encephalomyelitis [56].

Proregulatory DCs

Different subsets of DCs have been identified, which are markedly altered during helminth infection. In the MLN of *H. polygyrus*-infected mice, the proportion of $CD11c^{high}CD8\alpha^{intermediate}$ DCs declines in infection, indicating a reduced migration of cells from the lamina propria [141]. Moreover, there is a sharp increase in the proportion of tolerogenic $CD11c^{lo}$ DCs in the MLN; this cell type responds suboptimally to Toll-like receptor stimulation, is unable to prime a strong Th2 response from T cells, but induces much higher proportions of $CD4^+CD25^-$ to express Foxp3 than the conventional $CD11c^{hi}$ subset [142, 143].

This effect was mirrored in an in vitro setting when OVA-pulsed bone marrow-derived DCs (BMDCs) were cultured with HES and showed lower costimulatory molecule expression and cytokine output compared to untreated OVA-pulsed BMDCs [144]. These cells also induced IL-10-secreting $CD4^+CD25^+Foxp3^+$ cell generation from $CD4^+$ cocultures [144], indicating a potential regulatory pathway initiated by *H. polygyrus* products.

Vaccine-induced immunity

Irradiated *H. polygyrus* larvae given orally stimulate protection against subsequent challenge [62, 145–148]. Notably, the efficacy of this irradiated larval vaccine is diminished by the coadministration of unirradiated larvae, indicating that the development of adult worms is able to inhibit development and/or expression of protective immunity against subsequent reinfection [146, 147]. The ability of adult worms to suppress protective immunity was further demonstrated by vaccine failure in mice given irradiated larvae before or after receiving adult parasites by intraintestinal laparatomy [146] or oral gavage [149].

As well as infective (L3) stage larvae, live L4 larvae isolated on days 4 or 6 postinfection from the intestinal wall of donor-infected animals given subcutaneously, elicit an ~95–100 % reduction in worms present 3 weeks after challenge compared to unimmunized controls [150]. When immunization was performed with late-stage L3 (isolated 2 days postinfection) or L5 larvae (isolated 8 days postinfection) a lower degree of protective immunity was induced (~60 and ~70 % reduction respectively compared to unimmunized controls) [150].

Recently, an effective nonliving vaccine against *H. polygyrus* has been developed, in the form of total HES administered with alum adjuvant, which induced sterile immunity against infection [21]. Earlier work had shown that mice immunized with a 60,000 mol wt HES-derived glycoprotein isolated from HES prior to infection had lower egg burdens than control mice, indicating an antifecundity effect of vaccination with this component [151].

Molecular basis of chronic infection

Parasite excretory–secretory products

The ability of helminth parasites to persist in the host for many months or years, evading host immunity, is most likely due to the secretion of active immunomodulatory molecules [152]. The secretome of a parasite is likely to continually mediate interactions with the host, through direct contact with host cells in proximity to the worm, and potentially systemically. Helminth-secreted immunomodulators have been intensely studied, with some candidates now being tested for treatments of other diseases and as targets for antiparasite drugs [153].

Early investigation of HES found immunomodulatory factors that suppressed proliferation of mitogen-stimulated lymphocytes [154] and Th2-dependent antibody production to a bystander antigen through effects on T cells [155]. More recently, HES has been shown to display a wide range of immunomodulatory activities, including inhibiting activation of DCs [144, 156], induction of Tregs [132], and suppression of airway allergic inflammation [157].

The individual components of the complex HES mixture from adult worms have recently been defined through proteomic and sequencing technology with the identification of several hundred proteins in HES [5, 158]. Most prominent and numerous among the HES products are >20 members of the Venom allergen/*Ancylostoma* secreted protein-like (VAL) multigene family, which show extensive sequence variation between genes [5]. VALs have also been found to be highly immunodominant as indicated by recognition of primary and secondary infection serum, and monoclonal antibodies [21], although their function is as yet undefined [5, 158]. These studies also found acetylcholinesterases and proteases to be abundant in HES [5] along with apyrases, lipid-binding proteins, lysosymes, globins, and vitellogenin homologues [5, 158].

Stage and sex specificity of HES

A small number of studies have identified lifecycle-stage-specific expression patterns of certain HES antigens. Infective larval stages of *H. polygyrus* secreted the highest levels of proteolytic enzymes and acetylcholinesterase [159, 160], which may be involved in migration through host tissues directly after infection. Calreticulin has been shown to be highly expressed in L4 larval stages and is localized in areas associated with excretory–secretory processes [161]. A TGF-β homologue has been shown to be abundantly expressed in adults compared to larval stages, which may indicate an immunomodulatory function when the adults reside in the lumen of the intestine for long periods of time [162]. A limited number of sex-specific adult antigens in both HES and on the cuticle of the worms have also been found [163].

H. polygyrus, autoimmunity and allergy

The immunomodulatory properties of *H. polygyrus*, which extend far beyond the site of infection alone, have led to many investigations of the potential for and mechanisms of parasite downregulation of allergic and autoimmune conditions, as discussed below, as well as in the modulation of coinfections with other pathogens (reviewed in [1]).

Allergy

H. polygyrus offers protection in several murine models of allergy, including intestinal, airway, and cutaneous reactions. Mice fed peanut extract administered alongside the mucosal adjuvant cholera toxin produced peanut specific IgE had elevated plasma histamine levels and exhibited systemic anaphylactic shock symptoms. All phenotypes were diminished in *H. polygyrus*-infected mice [164]. In the presence of *H. polygyrus*, the peanut antigen-specific IL-13 levels were drastically reduced, and these dampened IL-13 levels along with protection from peanut allergy were lost when mice were treated with neutralizing IL-10 antibody [164]. The source of IL-10 and the mechanism by which it acts to dampen allergic responses to peanut antigen during *H. polygyrus* infection have not yet been determined.

H. polygyrus-infected mice had reduced inflammatory cell infiltrates and bronchoalveolar lavage eosinophilia in experimentally induced airway allergy to both ovalbumin [165–167] and the house dust mite antigen Der p 1 [166]. Protection against these allergens could be transferred by MLN cells from infected mice, which contained a high proportion of $CD4^+CD25^+Foxp3^+$ T cells, or by transfer of sorted $CD4^+CD25^+$ cells from infected mice, implicating the action of Treg cells in protection [166]. *H. polygyrus*-infected IL-$10^{-/-}$ mice were not protected from ovalbumin-induced asthma [165]; however, MLN cells transferred from IL-$10^{-/-}$ *H. polygyrus* infected mice could still protect from allergy to these antigens, suggesting that IL-10 independent mechanisms can confer protection from allergy [166].

Inflammatory bowel disease

Inflammatory bowel disease (IBD) is characterized by an inappropriate inflammatory response of the gut to microbial antigens. In humans, there are two main forms of the disease: Crohn's disease (CD), which can affect the entire length of the gut, and ulcerative colitis (UC), which is localized only to the colon. There are many differing mouse models of IBD (reviewed in [168]), and the effect of *H. polygyrus* infection on controlling the disease has been examined in a number of these models.

IL-$10^{-/-}$ mice suffer from spontaneous chronic colitis [169] associated with excessive IFN-γ production [170]. Spontaneous colitis develops sporadically over several months, but piroxicam treatment will induce rapid and uniform disease in IL-10-deficient mice [171, 172], which likely occurs as a result of increased colonic epithelial cell apoptosis causing a loss of barrier function to inflammatory microbial stimuli [172]. When *H. polygyrus* was given to piroxicam-treated IL-$10^{-/-}$ mice, the histological scores of colitis severity were drastically reduced within 14 days [173, 174]. LPMC from uninfected colitic mice released

the inflammatory cytokines IFN-γ, IL-12p40, and IL-17A, whereas LPMC from *H. polygyrus*-infected had significantly reduced levels of these cytokines [173, 174].

Severe colitis also develops when $RAG^{-/-}$ mice are reconstituted with $IL\text{-}10^{-/-}$ T cells and treated with piroxicam [175]. *H. polygyrus* colonization reduced gut inflammation in this model, as shown by lower levels of IFN-γ and IL-17 production by restimulated LPMC cells, and a drop in colonic histological score from an average of above 3 (some epithelial and muscle hypertrophy, mucus depletion, crypt abscesses, and epithelial erosions) to less than 1 (some mononuclear cell infiltrates in the lamina propria) [136, 176]. In $RAG^{-/-}$ mice that had been infected with *H. polygyrus*, and subsequently drug-cleared of the infection prior to transfer of the colitogenic $IL\text{-}10^{-/-}$ T cells and piroxicam administration, mice still showed reduced levels of inflammation compared to those that had never been infected [176]. The authors reported that protection coincided with downregulation of the costimulatory molecules CD80 and CD86 on DCs, thus inhibiting antigen presentation to T cells resulting in less inflammatory cytokine release [176].

TGF-βRII DN mice develop spontaneous colitis, which is unable to be suppressed by *H. polygyrus* infection [135]. The inability of *H. polygyrus* to suppress colitis in this model is likely due to the exacerbated IFN-γ levels seen in TGF-βRII DN mice, which are not dampened during *H. polygyrus* infection [135] and are known to aggravate intestinal inflammation.

Intrarectal injection of trinitrobenzene sulfonic acid (TNBS) administration also induces severe colitis in wild-type mice. When mice that had been infected with *H. polygyrus* for 10 days were given TNBS injection, they exhibited markedly reduced TNBS-induced colonic damage and inflammation and decreased Th1 cytokine mRNA expression compared to uninfected control mice, which was accompanied by increased IL-10 secretion during *H. polygyrus* infection [36, 177].

In contrast to other models of colitis, *H. polygyrus* seems to intensify colitis caused by the murine bacterial pathogen *Citrobacter rodentium* [178–180]. Disease exacerbation could be due to the influx of alternatively activated macrophages during *H. polygyrus* infection, which are less able to kill bacteria than classically activated macrophages [178], or due to increased IL-10 production by DCs impairing mechanisms that kill *C. rodentium*, leading to more persistent infection and colitis [180]. These studies exemplify the need to understand the causes of colitis, and the mechanisms by which helminths modulate disease progression, before helminth therapy can be applied to human inflammatory bowel diseases.

H. polygyrus is not the only parasitic nematode shown to have modulatory effects on the onset of colitis; both *T. spiralis* and excretory–secretory products from the hookworms *Ancylostoma caninum* and *Ancylostoma ceylanicum* can also ameliorate colitis progression in murine models, as reviewed in [168]. In human clinical trials, ova from the pig intestinal helminth parasite *Trichuris suis* reduced the severity of disease in some patients with UC and CD [181–183]. Although initial clinical trials with *T. suis* are promising, the fact that the therapy is not effective in 100 % of patients illustrates the need for further studies to understand the immunomodulatory actions of these helminths in murine models of IBD.

Type 1 diabetes

Nonobese diabetic (NOD) mice spontaneously become diabetic (as measured by blood glucose levels of ≥ 200 mg/dl) by 25 weeks of age [184, 185]. When these mice are infected with *H. polygyrus* at 5 weeks old, the onset of diabetes was completely blocked, at least until 40 weeks of age [184, 185]. Administering *H. polygyrus* when NOD mice were 7 and 12 weeks of age resulted in less effective protection from diabetes, yet onset was still delayed compared to untreated NOD mice [185]. The severity of insulitis (the infiltration of immune cells into the islets of Langerhans) was examined in mice aged 13 weeks, and was sharply reduced in NOD mice infected with *H. polygyrus* since the age of 5 weeks [185]. This reduction was maintained in *H. polygyrus* mice given anti-CD25 antibody [185], suggesting that *H. polygyrus* modulates type 1 diabetes (T1D) onset in a Treg-independent manner, although whether this protection extends beyond the 13-week time point has not been examined.

There is the possibility that the modulatory effects of *H. polygyrus* are due in part to changes in gut microbial composition during infection [186]. Studies to modify the microbial flora could address this, perhaps using fecal transplants, which would allow transfer of the microflora from *H. polygyrus* infected or naive mice to recipient mice using methods described in [187].

Role of the microbiota

H. polygyrus is localized in the anterior small intestine alongside a substantial microbial flora. The presence of specific species of bacteria within the gut is known to polarise naive T cells towards particular Th subset fates [188–190], and as the outcome of *H. polygyrus* infection is dependent on the immediate cytokine environment, it seems reasonable to imagine that commensal microbes may alter the ability of the murine immune system to cause worm expulsion.

Care must therefore be taken when performing experiments to compare the susceptibility to *H. polygyrus* in different mice, as the mice may initially differ in their microbial flora. Variation in microbial flora may be due to the source of mice, as mice of the same strain acquired from different vendors can

harbor different gut microbes [188], perhaps due to diet or housing conditions. The genotype of mice can also control microbial populations, as has been shown to be the case for MyD88-deficient mice, which have an altered microbial flora compared to MyD88-sufficient mice as MyD88 controls the release of some antimicrobial peptides [191].

After a 14-day infection with *H. polygyrus* in C57BL/6 mice, the abundance of *Lactobacillaceae* family members was increased in the ileum compared to naive mice [186]. It has yet to be demonstrated whether this shift is a helminth-mediated mechanism that acts to promote the survival of *H. polygyrus* within the murine host, or if it is simply as a consequence of a changing immune environment, in which bacteria of the *Lactobacillaceae* family are better able to survive. To resolve this, further studies to investigate the interplay among parasitic helminths, the microbial flora, and the immune system are necessary.

Conclusions and implications for human infections and disease

Parasitic helminth infections in humans and livestock are still responsible for unacceptably high levels of morbidity and economic loss worldwide. Understanding the mechanisms necessary for expulsion of the model gastrointestinal parasite *H. polygyrus* is likely to define new pathways, which target the immune system to provide the best protection against other helminth infections. The increasing prevalence of autoimmune diseases in the Western world correlates with the increasing absence of such helminth infections [192]. Our immune systems have evolved to develop in the presence of helminth parasite antigens, and it is vital to understand whether the human immune system can function optimally without this presence. Studies to isolate and understand how immunomodulatory factors secreted by helminths such as *H. polygyrus* act to maintain gut homeostasis are ongoing and will be invaluable both in understanding the interactions between helminths and the immune system and in the development of new pharmaceutical therapies for autoimmune and allergic diseases worldwide.

Acknowledgments We thank the Wellcome Trust for support through a studentship to LAR and programme grant funding to RMM and the Medical Research Council/UCB Celltech for CASE studentship funding to KJF.

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J Immunol 2011; 187:4764-4777; Prepublished online 30 September 2011;
doi: 10.4049/jimmunol.1004140
http://www.jimmunol.org/content/187/9/4764

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Heligmosomoides polygyrus Elicits a Dominant Nonprotective Antibody Response Directed against Restricted Glycan and Peptide Epitopes

James P. Hewitson,*[,1] Kara J. Filbey,*[,1] John R. Grainger,*[,2] Adam A. Dowle,[†] Mark Pearson,* Janice Murray,* Yvonne Harcus,* and Rick M. Maizels*

Heligmosomoides polygyrus **is a widely used gastrointestinal helminth model of long-term chronic infection in mice, which has not been well-characterized at the antigenic level. We now identify the major targets of the murine primary Ab response as a subset of the secreted products in *H. polygyrus* excretory–secretory (HES) Ag. An immunodominant epitope is an O-linked glycan (named glycan A) carried on three highly expressed HES glycoproteins (venom allergen *Ancylostoma*-secreted protein-like [VAL]-1, -2, and -5), which stimulates only IgM Abs, is exposed on the adult worm surface, and is poorly represented in somatic parasite extracts. A second carbohydrate epitope (glycan B), present on both a non-protein high molecular mass component and a 65-kDa molecule, is widely distributed in adult somatic tissues. Whereas the high molecular mass component and 65-kDa molecules bear phosphorylcholine, the glycan B epitope itself is not phosphorylcholine. Class-switched IgG1 Abs are found to glycan B, but the dominant primary IgG1 response is to the polypeptides of VAL proteins, including also VAL-3 and VAL-4. Secondary Ab responses include the same specificities while also recognizing VAL-7. Although vaccination with HES conferred complete protection against challenge *H. polygyrus* infection, mAbs raised against each of the glycan epitopes and against VAL-1, VAL-2, and VAL-4 proteins were unable to do so, even though these specificities (with the exception of VAL-2) are also secreted by tissue-phase L4 larvae. The primary immune response in susceptible mice is, therefore, dominated by nonprotective Abs against a small subset of antigenic epitopes, raising the possibility that these act as decoy specificities that generate ineffective humoral immunity.** ***The Journal of Immunology*****, 2011, 187: 4764–4777.**

Heligmosomoides polygyrus is a widely used experimental mouse model for the highly prevalent human and animal gastrointestinal helminth infections (1, 2). This system has provided major new findings in parasite immunology (3, 4), immune regulation (5, 6), nutrition (7), and ecology (8), and yet little information is available on the specific parasite Ags to which the host immune system is exposed. In this study, we set out to identify the molecular targets of murine humoral Abs, to define individual *H. polygyrus* Ags, and to investigate the role of major Ab specificities in the host–parasite relationship.

Among the interesting facets of *H. polygyrus* is its ability to establish a chronic infection in most strains of laboratory mice, with the genetic background influencing the rate of expulsion rather than susceptibility per se (9–11). Genetically resistant mice mount a more rapid serum Ab response measured against adult worm somatic extract (12) or excretory–secretory (ES) Ags from cultured adult parasites (13, 14), and immunity to reinfection is compromised in B cell-deficient mice (4, 15–17). Early investigations had reported that passive transfer of serum from infected mice can confer a degree of immunity to *H. polygyrus* both in terms of worm number and in fecundity (18); this effect was associated with IgG1 isotype Abs (19, 20). More recently, IgG1 serum Abs have been demonstrated to reduce the fecundity and viability of adult worms and shown to require affinity maturation to confer any resistant effect (15).

As has been recently pointed out (21), in current nematode model systems, few serologically important Ags have yet been identified. Previous studies have relied on either crude whole-worm homogenates or collected secreted products as a more restricted but nevertheless complex antigenic set. Therefore, we decided to analyze the humoral Ab response to *H. polygyrus* in terms of specific Igs, to define the molecular targets of parasite-specific Abs, and to test whether these played any protective role against the infection in vivo. We tested Ab reactivities both to crude parasite antigenic extracts and also to preparations collected from in vitro culture of adult worms, termed "excretory–secretory" (ES) Ags, which are strongly implicated in immunomodulation of the host (6, 22). We report in this article that several major constituents are homologs of venom allergen *Ancylostoma*-

*Institute of Immunology and Infection Research, University of Edinburgh, Edinburgh EH9 3JT, United Kingdom; and [†]Technology Facility, Department of Biology, University of York, York YO10 5YW, United Kingdom

[1]J.P.H. and K.J.F. are joint first authors.

[2]Current address: Mucosal Immunology Unit, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes for Health, Bethesda, MD.

Received for publication December 21, 2010. Accepted for publication August 24, 2011.

This work was supported by a Wellcome Trust Programme grant and a Wellcome Trust Ph.D. studentship (to J.R.G.). K.J.F. is supported by a Medical Research Council Collaborative Awards in Science and Engineering studentship through UCB Celltech.

Address correspondence and reprint requests to Dr. Rick M. Maizels, Institute of Immunology and Infection Research, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, United Kingdom. E-mail address: rick.maizels@ed.ac.uk

The online version of this article contains supplemental material.

Abbreviations used in this article: ASP, *Ancylostoma*-secreted protein; ES, excretory–secretory; ESI, electrospray ionization; HES, *Heligmosomoides polygyrus* adult excretory–secretory products; HEx, *Heligmosomoides polygyrus* adult somatic extract; HM-65, high molecular mass 65 kDa antigenic complex; IP, immunoprecipitation; LC, liquid chromatography; MLN, mesenteric lymph node; MS/MS, tandem mass spectrometry; PC, phosphorylcholine; PNGase, peptide:N-glycanase; TFH, T follicular helper cell; TFMS, trifluoromethanesulfonic acid; VAL, venom allergen *Ancylostoma*-secreted protein-like.

secreted protein (ASP)-like (VAL) Ags related to the vaccine candidates of human and canine hookworms (23, 24). However, the response to infection is dominated by anti-glycan specificities, and the murine Ab profile is highly restricted with respect to the range of Ags recognized.

Materials and Methods

Parasites, Ags, and mice

The original stock of *H. polygyrus bakeri* used in these studies was kindly supplied to us by Prof. J.M. Behnke (University of Nottingham, Nottingham, U.K.). Parasites, *H. polygyrus* ES (HES) Ag and adult worm somatic extract (HEx) were produced as described previously (6, 25, 26). Day 5 fourth-stage larvae were collected from the intestinal wall of infected mice and ES collected over a 3-d culture period in the same manner as adult HES. Female C57BL/6 and BALB/c mice (6–10 wk old) were bred in-house, and animal studies were performed under U.K. Home Office License. Mice were infected with 200 *H. polygyrus* L3 by oral gavage, and fecal egg counts and adult worm burdens were determined by standard procedures (2). For secondary infection, mice were treated orally with pyrantel embonate (27) in the form of 2.5 mg Strongid P paste in 0.2 ml water on days 28 and 29 postprimary infection. Drug-treated mice were rechallenged with 200 L3 by gavage 2 wk later. Where indicated, HES was heat denatured by incubating at 95˚C for 20 min (6).

One-dimensional and two-dimensional gel electrophoresis and Western blotting

HES and HEx (1–10 μg) were separated, silver stained, or blotted as described previously (28). Blots were blocked in 2% BSA–TBS with 0.05% Tween 20 (TBST) for 2 h at room temperature, before being probed with sera (1/500 dilution) or mAbs (2 μg/ml) at 4˚C overnight. Following extensive washing in TBST, blots were incubated with HRP-conjugated secondary Abs (anti-mouse Ig 1/2000, Dako P0460, DakoCytomation; anti-mouse IgM 1/1000, Southern Biotech 1020-05, and anti-mouse IgG1, 1/2000 Southern Biotech 1070-05; Southern Biotechnology Associates) for 1 h at 37˚C, washed in TBST, and then developed as described previously (28). Alternatively, IgA blots were incubated with biotinylated anti-mouse IgA (1/500, M31115; Invitrogen), followed by HRP–streptavidin (1/2000; Sigma-Aldrich), and developed as above. Mouse IgM mAb Bp-1 (29) was used for anti-phosphorylcholine (PC) blots at 1/1000 dilution and detected with anti-mouse Ig as above.

ELISA

HES and HEx (1 μg/ml) were coated on Immunoplates (Nunc) in 0.06 M carbonate buffer overnight (4˚C), blocked with block solution (2% BSA–TBST) for 2 h (37˚C), and then incubated with doubling dilutions of sera (in block solution) for 2 h (37˚C). For comparison of HES and L4 ES, each were used to coat plates at a range of dilutions. Worm-specific Ab titers were detected using the secondary reagents described above and developed with ABTS (Insight Biotech). Titer was determined as the reciprocal dilution at which the sample dropped below background levels. For anti-PC ELISA, plates were coated with 1 μg/ml PC-conjugated BSA (30), serum was used at a 1/500 dilution and mAb at 5 μg/ml. Anti-*H. polygyrus* mAbs were used at 5 μg/ml for all ELISAs, unless stated. Goat anti-rat Ig (1/2000, Dako P0450; DakoCytomation) was used as a secondary for experiments with rat sera.

mAb and polyclonal Ab production

For mAbs, spleens and mesenteric lymph nodes (MLN) were recovered from C57BL/6 mice at day 28 postinfection and fused with SP2 cells. Fused cells were cultured for 12–14 d in HAT selection media (RPMI 1640

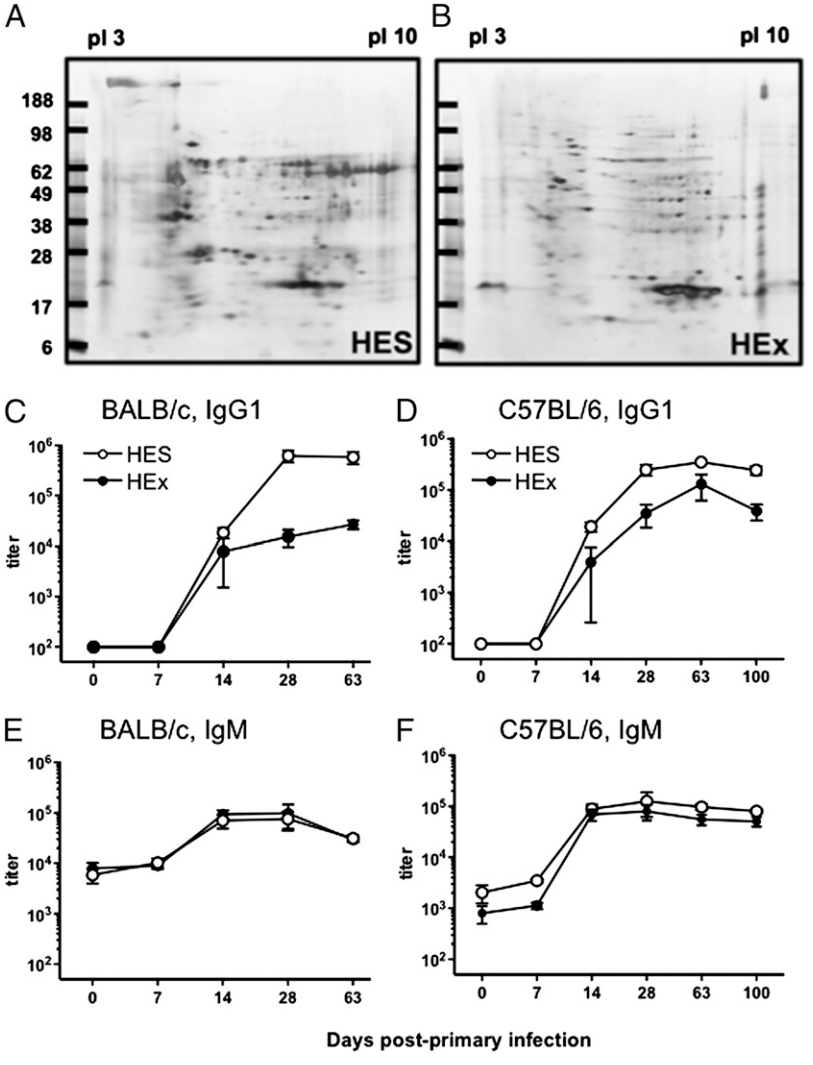

FIGURE 1. The predominant serological Ags of *H. polygyrus* are in HES rather than somatic extract. *A* and *B*, Two-dimensional silver-stained gels of 10 μg HES (*A*) and Hex (*B*). Molecular mass markers (in kDa) are indicated on the left. *C* and *D*, Anti-HES (open symbols) and HEx (solid symbols) IgG1 serum Ab titres from BALB/c (*C*) and C57BL/6 (*D*) mice at various time points following primary infection. *E* and *F*, Anti-HES and HEx IgM serum Ab titres in the same BALB/c (*E*) and C57BL/6 (*F*) mice. Each point represents the mean and SEM of data from five individual mice separately assayed. Data are representative of two independent experiments.

medium supplemented with 20% FCS [Hyclone], 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine [all Life Technologies], HAT [100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine] and OPI [1 mM oxaloacetate, 0.45 mM pyruvate, and 0.2 U/ml insulin; all Sigma-Aldrich]). Plates were ELISA screened for the production of anti-HES Abs as above, and positive wells were cloned by two to three rounds of limiting dilution. Cells were then adapted into standard complete RPMI 1640 medium (RPMI 1640 medium supplemented with 10% FCS and the above concentrations of penicillin, streptomycin, and L-glutamine) and grown in bulk cultures in Vectra Cell Bioreactors (Bio-Vectra) to produce mAb. Ab isotypes were determined either with a mouse Ab isotype kit (Isostrip; Roche) or the anti-mouse Ig secondary Ab described above. Abs were purified using an AKTA prime fast protein liquid chromatography (LC) with a HiTrap protein G HP (IgG1 mAb) or HiTrap IgM purification (IgM and IgA) column, according to the manufacturer's instructions, and then dialyzed extensively into PBS. For the rat polyclonal Abs, rats were immunized with 25 μg HES or recombinant *H. polygyrus* calreticulin (AM296015 (31), produced in *Escherichia coli*) in alum adjuvant i.p., then boosted with 10 μg Ag on days 28 and 35, before serum collection on day 42. The mouse IgG1 myeloma MOPC 31C from American Type Culture Collection was used as an isotype control. A hybridoma-producing mouse anti-DNP IgM control was produced as above from mice immunized with 50 μg DNP-conjugated keyhole limpet hemocyanin in alum adjuvant i.p. and then boosted on days 12 and 13 with 1 μg DNP–keyhole limpet hemocyanin in PBS i.v. before spleen harvest on day 14. Screening was performed using DNP–OVA and anti-mouse IgM secondary Ab as above. Hybridomas were also produced from mice immunized with 25 μg HES in IFA (Sigma-Aldrich) i.p. then boosted on days 48 and 49 with 1 μg HES in PBS i.v. before spleen harvest and fusion on day 50.

Immunoprecipitation

HES was labeled with biotin (~40 μg biotin reagent/100 μg HES) using EZ-link Sulfo-NHS Biotinylation kit (Pierce) for 2 h on ice and then dialyzed overnight into PBS. Biotinylated HES was then precleared with protein G-agarose beads (16-266; Millipore) in the presence of MOPC 31C IgG1 isotype control for 30 min at room temperature. Unbound HES (2 μg) was then incubated with 2 μg various anti-HES IgG1 mAb, MOPC IgG1 control, or 5 μl C57BL/6 day 28 primary or day 14 secondary infection sera, in nondenaturing immunoprecipitation (IP) buffer (20 mM Tris [pH 8], 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 1% Triton X-100) for 2 h, and then with protein G-agarose beads overnight at 4˚C with rotation. Beads were then washed 5 × 5 min in IP buffer, and bound proteins were eluted with 0.1 M glycine (pH 2.7). Eluted proteins were buffer exchanged into PBS (with MicroBio-Spin 6 chromatography columns; Bio-Rad), run on one-dimensional and two-dimensional gels and Western blotted as described above, and then probed with 1/2000 streptavidin-horseradish peroxidase (Sigma-Aldrich) before developing to allow visualization of biotinylated proteins.

Deglycosylation

For peptide:N-glycanase (PNGase) F treatment, HES or RNase B (Sigma-Aldrich) was denatured by heating to 95˚C for 5 min in the presence of 0.1% SDS and 100 mM 2-ME, before the addition of 10 U PNGase F (Sigma-Aldrich) and 1% Triton X-100. Samples were incubated at 37˚C for 3 h. For PNGase A treatment, HES was diluted in 100 mM ammonium bicarbonate (pH 8.5), heat denatured, and digested overnight with 1 μg proteomics grade trypsin at 37˚C (Sigma-Aldrich), after which trypsin was inactivated by boiling. Complete digestion of HES was confirmed by SDS-PAGE analysis (data not shown). Tryptic peptides were then lyophilized and resuspended in 100 mM sodium acetate (pH 5) and treated with 0.2 mU PNGase A (Roche) for 24 h at 37˚C. For subsequent ELISA analysis, peptides were bound to plates in carbonate buffer at 1 μg/ml and then tested for Ab binding as above. For PNGase F and A digestions, control proteins were treated in an identical manner but in the absence of enzyme. For chemical deglycosylation of HES with trifluoromethanesulfonic acid (TFMS), HES, or RNase B were dialyzed into 0.1 M ammonium acetate then lyophilized until completely dry. The pellet was resuspended in 10% anisole in TFMS (both Sigma-Aldrich) at 4˚C, and the reaction was allowed to proceed for 2 h before neutralization with a 60% pyridine solution in a methanol–dry ice bath. The soluble fraction of HES was dialyzed into PBS, and the residual precipitate was solubilized in 1% SDS.

Affinity purification of VAL proteins

Twenty milligrams each of anti–VAL-1 (4-M15), 2 (4-S4), and 4 (2-11) mAbs were dialyzed into coupling buffer (0.1 M sodium bicarbonate and 0.5 M NaCl [pH 8.4]) and then reacted with swollen cyanogen bromide-activated Sepharose beads (Sigma-Aldrich) overnight at 4˚C with rotation. Unreacted groups were blocked with 0.2 M glycine (pH 8) for 2 h at room temperature, after which the beads were washed in five cycles of coupling

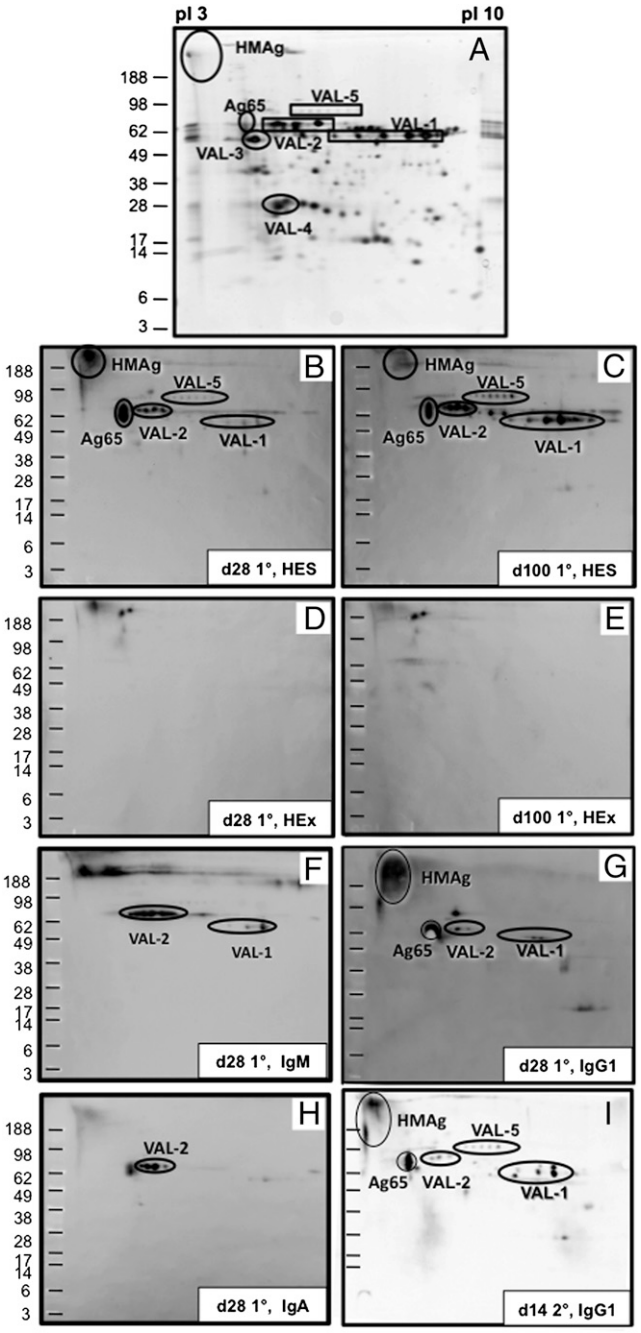

FIGURE 2. Polyclonal Abs recognize a restricted set of *H. polygyrus* Ags by Western blot. *A*, Silver-stained two-dimensional gel of HES indicating major components identified by mass spectrometry (34). HMAg and Ag65 indicate the position of Ags (subsequently termed HM-65; see Fig. 3*D*) that do not stain with silver. *B* and *C*, Two-dimensional Western blot of HES with sera from C57BL/6 mice taken at day 28 (*B*) and day 100 (*C*) following primary infection with *H. polygyrus*, developed with a polyvalent anti-Ig conjugate. *D* and *E*, As above, but with HEx. *F*, Two-dimensional Western blot of anti-HES IgM in sera from 28-d infected C57BL/6 mice. *G*, As above, for anti-HES IgG1. *H*, As above, for anti-HES IgA. *I*, Two-dimensional Western blot of anti-HES IgG1 in sera C57BL/6 mice collected 14 d following a secondary challenge infection. For each assay, sera were pooled from five mice. Molecular mass markers are indicated in kilodaltons, and results are representative of two or more experiments. Naive mouse sera showed no positive binding under the same conditions (data not shown).

buffer, followed by 0.1 M sodium acetate and 0.5 M NaCl (pH 4). For affinity purification, 10 μg HES was treated as before for IP, eluted proteins were run on one-dimensional SDS-PAGE, and bands of interest were excised for mass spectrometry analysis.

Sequence database and mass spectrometry

In studies to be published elsewhere, a database compiled of ~466,000 Roche 454 sequence reads from normalized and non-normalized adult *H polygyrus* cDNA was assembled into ~20,000 predicted gene products (Y. Harcus, J.P. Hewitson, K.J. Filbey, J.R. Grainger, M. van Agtmaal, M. Thompson, N. Wrobel, S. Bridgett, M.L. Blaxter, and R.M. Maizels, manuscript in preparation). This database was used to match peptides identified by mass spectrometry of SDS-PAGE and two-dimensional gel-purified proteins. The individual protein genes described in this article (VAL-1, VAL-2, VAL-3, VAL-4, VAL-5, and VAL-7) were each amplified by PCR from *H. polygyrus* mRNA using gene-specific primers, and multiple independent clones were sequenced to verify the sequences predicted by the assembly algorithm. The full sequences for VAL-1 to VAL-5 and VAL-7 have been deposited with the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) under accession numbers JF914902, JF914906, JF914909, JF91410, JF914911, and JF914913, respectively. Immunoprecipitated HES proteins were prepared for mass spectrometry analysis as described previously (28). Positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron mode, equipped with an Nd:YAG smart beam laser. MS spectra were acquired over a mass range of *m/z* 800–4000, and monoisotopic masses were obtained using a SNAP (sophisticated numerical annotation procedure) averaging algorithm. The 10 strongest peaks of interest, with an signal/noise ratio >30, were selected for tandem mass spectrometry (MS/MS) fragmentation in LIFT mode. Bruker flexAnalysis software (version 3.3) was used to perform the spectral processing and peak list generation for both the MS and MS/MS. Identification of mAb 2-11 target VAL-4 required LC-electrospray ionization (ESI)-MS/MS using an Ultimate nanoLC system (Dionex) equipped with a PepMap C_{18} trap (300 μm × 0.5 cm, Dionex) and an Onyx C_{18} monolithic silica capillary column (100 μm × 15 cm; Phenomenex). Peptides were eluted over with a acetonitrile gradient (solvent A = 2% [v/v] acetonitrile, 0.1% [v/v] formic acid in H_2O; solvent B = acetonitrile, 0.1% [v/v] formic acid; gradient conditions consisted of 3 min solvent A), and then, a linear 0–50% gradient of solvent B over 20 min was applied, followed by a 50-min wash at 95% solvent B. The flow rate was 1.2 μl/min, and the column temperature was 60°C. The nanoLC was interfaced with a high capacity trap ultra ETD II ion-trap LC-MS/MS system (Bruker Daltonics) with an online nanoESI source. Positive ESI-MS and MS/MS were acquired using AutoMSn mode, over the 300- to 1800-*m/z* range. Instrument control, data acquisition, and processing were performed using Compass 1.3 SP1 software (Esquire control, Hystar, and DataAnalysis; Bruker Daltonics). MS/MS data were submitted to database searching against an in-house database containing the predicted gene products using the Mascot program (Matrix Science, version 2.1), through the Bruker ProteinScape interface (version 2.1). Database searching was run with a peptide tolerance of 250 ppm and MS/MS tolerance of 0.5 Da. Peptide matches with expect values <0.05 at a Mowse significance threshold of $p < 0.05$ were considered significant.

Immunofluorescence

For sections, adult *H. polygyrus* worms were snap-frozen on dry ice in Cryo-M-Bed mountant (Bright Instruments). Cryostat sections (5 μm; Leica) were cut onto Polysine slides (VWR), dried, and then fixed in 100% acetone for 10 min. Sections were washed twice with PBS for 10 min and then incubated with the various mAb (50 μg/ml in PBS containing 1% FCS) for 2 h at room temperature, washed twice in PBS as before, and then incubated with secondary anti-mouse Ig tetramethylrhodamine isothiocyanate (1/100 in PBS) for 1 h at room temperature. Following extensive washing, sections were mounted in anti-fade Vectashield mountant (Vector Laboratories). Staining was visualized with an Olympus fluorescent microscope. Non-fixed intact worms were stained on ice in round-bottom 96-well plates and then treated as above.

Radiolabeling of adult worm surface

Adult *H. polygyrus* were surface radiolabeled essentially as described in earlier publications (32, 33). Eppendorf tubes (1.5ml) were coated with 200 μl of a 1 mg/ml solution of Iodination reagent (Pierce) in chloroform. Once dried, the tubes were washed several times with PBS, before transfer of ~500 adult worms and 500 μCi [^{125}I] (PerkinElmer) on ice. The sample was incubated with frequent agitation for 10 min and then quenched by the addition of a saturated solution of *L*-tyrosine (Sigma-Aldrich). Radiolabeled parasite surface material was then produced as described above as with HEx, except parasites were homogenized in 1.5% n-octyl glucoside detergent and 1% protease inhibitor mixture (P8340; Sigma-Aldrich). Immunoprecipitates were performed as above (anti–HM-65 mAb 9.1.3 or rat anti-HES polyclonal Ab) or with anti-mouse IgM-agarose (A4540; Sigma-Aldrich). Autoradiographs were carried out on dried gels as described previously (32, 33).

Vaccination and passive immunization

C57BL/6 females were immunized with 25 μg HES in alum adjuvant i.p., then boosted on days 28 and 35 with 5 μg HES–alum i.p. Mice were challenged with 200 *H. polygyrus* L3 larvae, fecal egg counts were determined at days 14 and 28 postinfection, and adult worms counted at day 28. For passive immunization, C57BL/6 females were treated on day −1 and then every 2–3 d postinfection (with 200 *H. polygyrus* L3 larvae) with either 0.2 or 1 mg mAb i.p. (for IgG1 mAbs) or i.v. (for IgM mAbs) as detailed in the figure legend. Eggs and worm numbers were determined as above.

Results

Ab responses are predominantly directed at secreted, rather than somatic, parasite Ags

As a first step in defining immunogenic products of adult *H. polygyrus*, we compared a conventional antigenic preparation comprising a soluble whole parasite extract (HEx), with products released by live parasites maintained in serum-free tissue culture medium (HES). The overall protein compositions of HES and HEx are very different, as shown in Fig. 1*A* and 1*B*, respectively, and confirmed by recent proteomic analysis (34).

As IgG1 Abs predominate and have a protective role in both primary and secondary *H. polygyrus* infection (15, 19, 20), we then compared IgG1 responses to HES and HEx following a primary infection. Ag-specific IgG1 responses to HES were detected by ELISA in both BALB/c and C57BL/6 mice by day 14 postinfection and by day 28 reached a high titer, which was main-

Table I. mAbs to HES Ags

Ag, Specificity	Clone	Source	Isotype
Glycan A (VAL-1-2-5)	13.1, 2-2, 2-12, 2-13, 2-62, 3-8, 3-11, 3-28, 3-29, 3-40, 3-42, 3-55	d28 SPL	IgM
Glycan B (HM-65)	14.3	d28 SPL	IgA
	3-31	d28 SPL	IgM
	4-M9	d28 MLN	IgM
	4-M7, 4-M17	d28 MLN	IgG1
	9.1.3	HES/IFA	IgG1
VAL-1	2-6, 3-6, 3-10, 3-38, 3-39	d28 SPL	IgG1
	4-M4, 4-M15, 4-M20, 4-M23, 4-M25	d28 MLN	IgG1
VAL-2	4-S4	d28 SPL	IgG1
VAL-4	2-11	d28 SPL	IgG1

d28 SPL, day 28 spleen.

tained for at least 63 (BALB/c; Fig. 1*C*) and 100 d (C57BL/6; Fig. 1*D*). Moreover, IgG1 titers to HES were up to 20-fold higher than those to HEx and showed less variation between individual animals. We therefore focused in the majority of subsequent Ab investigations on HES.

The anti-parasite IgM response differed from the IgG1 response in several regards (Fig. 1*E*, 1*F*). Significant background levels of both anti-HES and anti-HEx IgM Abs were noted in naive mice, and following infection, specific IgM titers reached a relatively early plateau (day 14). Furthermore, reactivity to HES and HEx was equivalent at all time points. We were unable to detect the target of (presumably natural) IgM Abs present in naive mice using both Western blot and mAb techniques, perhaps because of their relatively weak affinity (data not shown).

Ag specificity of polyclonal Ab responses

To identify individual antigenic targets of the Ab responses, we adopted both polyclonal and monoclonal strategies. First, we used two-dimensional SDS-PAGE separation of HES Ags, by which ~100 distinct protein spots are observed (Figs. 1*A*, 2*A*); the identity of most major proteins has been determined by mass spectrometry (34). Despite the abundance of potential Ags, however, the two-dimensional Western blot profile of polyclonal sera from C57BL/6 mice with 28-d primary infection is much more

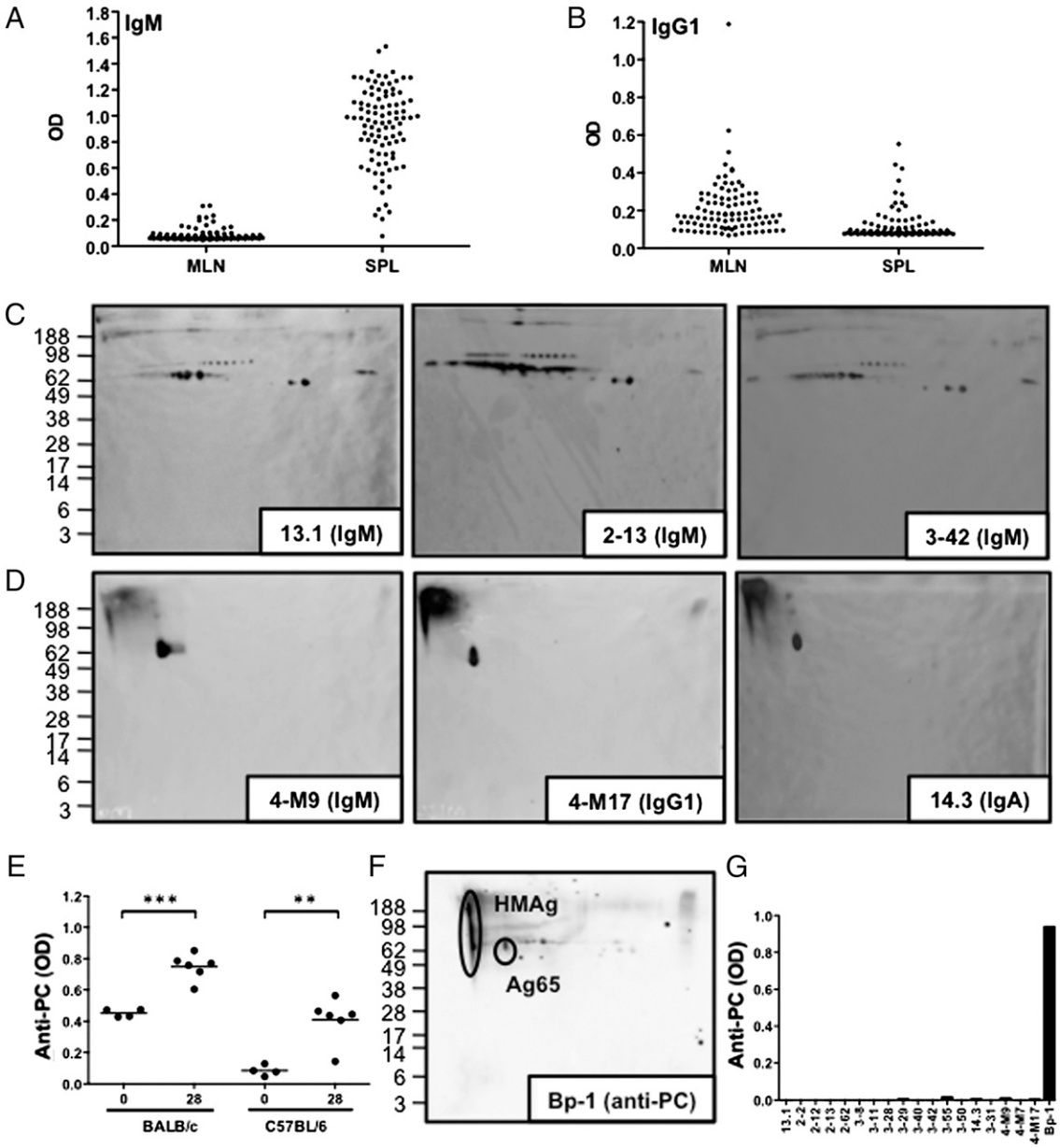

FIGURE 3. HES-specific mAbs from mice infected with *H. polygyrus*. *A* and *B*, Frequency of HES-specific hybridoma cells producing IgM (*A*) or IgG1 (*B*) from the MLNs and spleens of infected C57BL/6 mice at day 28 postinfection, as determined by ELISA. Each dot represents a single well of a 96-well plate. *C*, Representative two-dimensional Western blots of three different IgM anti-HES mAbs derived from infected mice, which bind to glycan A. *D*, Representative two-dimensional Western blots of anti-HES IgM, IgG1, and IgA mAbs recognizing glycan B on the HM-65 Ags. *E*, Anti-PC Abs measured by ELISA against PC-BSA in naive and 28-d infected sera from BALB/c and C57BL/6 mice. *F*, Two-dimensional Western blot of HES probed with anti-PC mAb Bp-1. The positions of the HM-65 Ags are circled. *G*, Reactivity to PC measured by ELISA on BSA–PC-coated plates; data from a panel of anti-HES mAbs are shown, together with Bp-1–positive control. Molecular mass markers are indicated in kilodaltons. $^{**}p < 0.01$, $^{***}p < 0.001$.

restricted (Fig. 2*B*). These were identified by proteomics as members of the venom allergen/VAL family of proteins (35, 36), specifically VAL-1, VAL-2, and VAL-5 (Fig. 2*A*) (34). Additional reactivity was noted to both a high molecular mass component and a smaller 65-kDa Ag, which do not appreciably silver stain and did not give measureable peptides for proteomic analysis; we term this Ag combination HM-65 (see below).

As reactivity increases over a long time frame, and as the C57BL/6 mouse is considered to be a slow responder to infection (37, 38), we also examined the serological response at 100 d postinfection (Fig. 2*C*); at this time point, responses to all the Ags previously noted were substantially stronger, but there remained a restricted repertoire of target Ags.

Consistent with ELISA results indicating that anti-HEx somatic extract responses were relatively weak, the same sera showed only slight reactivity by Western blot (Fig. 2*D*, 2*E*). The primary anti–VAL Ab response to HES was predominantly of the IgM isotype, although a smaller amount of anti–VAL-2 IgA was also noted (Fig. 2*F*, 2*H*). In contrast, anti-HES IgG1 response was detected against HM-65 (Fig. 2*G*). Polyclonal IgE did not provide a measurable signal by Western blot (data not shown).

We also examined the secondary response mounted by genetically susceptible mice cleared of infection by chemotherapy; in these mice, protective immunity is stimulated against challenge infection (9, 39). Importantly, immunity to challenge infection is ablated in B cell- or Ab-deficient mice (4, 15, 16, 40). By Western blot analysis, secondary IgG1 Abs showed a similar profile to samples from primary infection, albeit with a 10- to 30-fold higher titer (data not shown) and correspondingly stronger binding patterns (Fig. 2*I*). In contrast, secondary IgM responses were similar to primary Abs with respect both the titer and specificity profile (data not shown).

mAb specificities

We next generated a panel of monoclonal Abs to dissect the antigenic response in fine detail, using spleens and draining MLN from infected mice at day 28 (Table I). Despite taking cells at this relatively late time point in the primary response, a substantial proportion of monoclonals represented Abs that had not undergone class switch from the IgM isotype, particularly when splenocytes rather than MLN cells were used (Fig. 3*A*). The great majority (12 of 14) of these IgM mAbs displayed a similar Western blot profile against HES (Fig. 3*C*) and bound to proteins previously identified as VAL-1, VAL-2, and VAL-5 by proteomic analysis. In fact, this indicates that the observed specificity of polyclonal serum (Fig. 2*B*, 2*C*) can be largely replicated by a single (monoclonal) Ab specificity and that an immunodominant epitope is shared by at least three members of the VAL family.

A different pattern was shared by a smaller number of Abs, including IgM, IgG1, and IgA isotypes, which bound to HM-65 (Fig. 3*D*). Notably, these components do not stain with silver and do not give measurable peptides for proteomic analysis, mimicking the pattern seen with polyclonal IgG1 from primary infections (Fig. 2*G*).

Because of the prominence of PC in many helminth products, including high molecular mass species with nonproteinaceous composition (30, 41), we tested for PC reactivity in infection sera and for PC epitopes in HES. Serum Abs from *H. polygyrus*-infected mice showed a modest degree of anti-PC binding (Fig. 3*E*), as has been reported previously (42). When two-dimensional Western blots were probed with monoclonal anti-PC Ab, the major positive reaction was with HM-65 (Fig. 3*F*). Despite this, none of the anti-HES monoclonals bound directly to PC (Fig. 3*G*), indicating that they target a non-PC specificity.

IgG Abs recognize heat-labile epitopes of secreted VAL Ags

The majority of IgG1 mAbs raised from infected mice (12 of 14) failed to react with HES by Western blot, despite their strong reactivity to native HES by ELISA (Fig. 4*A* and data not shown). We interpreted this to mean that the IgG1 response is predominantly directed against conformational protein epitopes, which

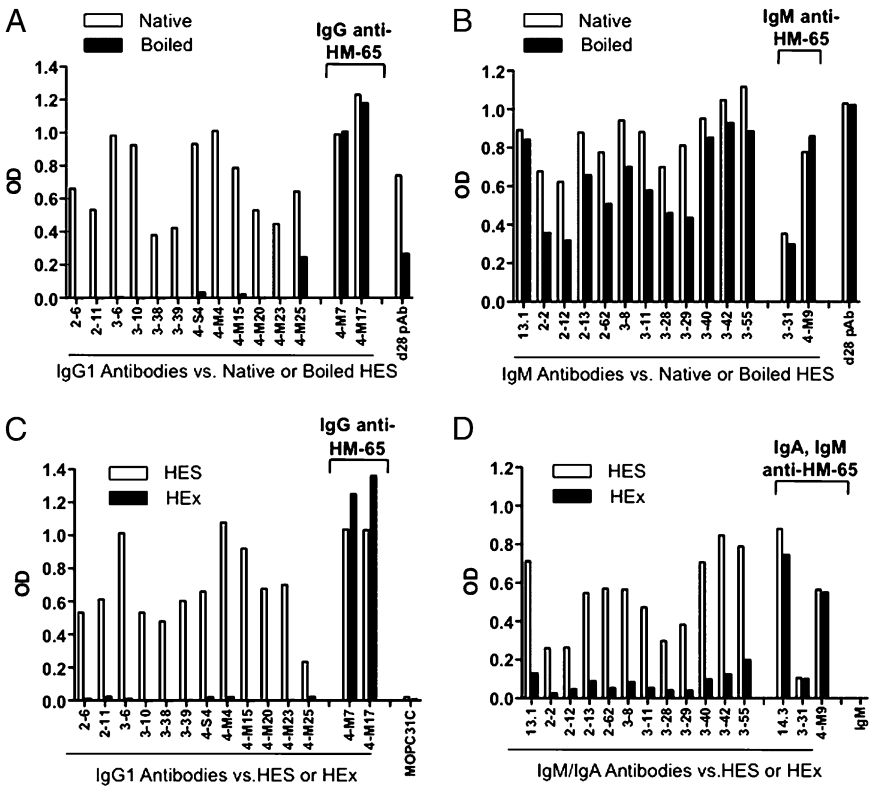

FIGURE 4. Most HES-specific IgG1 Abs recognize heat-labile epitopes absent from parasite extract. *A*, ELISA reactivity of anti-HES IgG1 mAbs to native (□) and boiled (■) HES. Polyclonal sera from 28-d infected C57BL/6 mice were included as a positive control. *B*, As above, for IgM mAbs. *C*, ELISA reactivity of anti-HES IgG1 mAbs to HES (□) and HEx (■). MOPC 31C myeloma IgG1 was included as a negative control. *D*, ELISA reactivity of anti-HES IgM and IgA mAbs to HES (□) and HEx (■). Anti-DNP IgM mAb was included as a negative control.

are destroyed following detergent denaturation during SDS-PAGE. In support of this, although IgM monoclonals are equally reactive to native or heat-denatured HES (Fig. 4*B*), heat denaturation of HES ablated all IgG1 mAb ELISA reactivity, with the exception of the two anti–HM-65 IgG1 Abs (Fig. 4*A*). Furthermore, although most IgG1 mAbs (Fig. 4*C*) and IgM mAbs (Fig. 4*D*) show little reactivity to somatic extract (HEx), all Abs specific for HM-65 show equally strong binding to HES and HEx.

To identify the heat-labile determinant(s) recognized by the IgG1 mAbs, an immunoprecipitation strategy was used using biotin-labeled nondenatured HES and protein G beads. The majority of these IgG1 monoclonals (10 of 14) were specific for a band that migrates in the position of VAL-1, as represented by 4-M15 (Fig. 5*A*). Two additional mAbs immunoprecipitated Ags comigrating with VAL-2 (4-S4) and VAL-4 (2-11).

Two-dimensional analysis of Ags immunoprecipated by day 28 polyclonal infection serum confirmed that the dominant target of primary IgG Ab in C57BL/6 mice was VAL-1 (Fig. 5*D*), as well as a spot that comigrates with another VAL protein abundant in HES, VAL-3 (Fig. 2*A*). Moroever, secondary IgG immunoprecipitated two further homologs, VAL-4 and VAL-7 (Fig. 5*E*). Comparison of profiles from Western blot (Fig. 2*G*, 2*I*) and immunoprecipitation (Fig. 5*D*, 5*E*) indicates that Western blotting gives an incomplete picture of IgG1 Ab specificity, as a result of the predominantly conformationally dependent VAL epitopes, whereas immunoprecipitation of biotinylated Ags omits the HM-65 group of Ags with low protein content.

To formally identify the immunoprecipitated proteins, samples bound by each Ab were eluted from one-dimensional gel bands (Fig. 5*F*) and subjected to mass spectrometry, matching the respective protein sequences (Fig. 5*G*). These assays also indicate that VAL-1, VAL-2, and VAL-4 do not interact with other proteins present in HES, given the absence of any coimmunoprecipitated components.

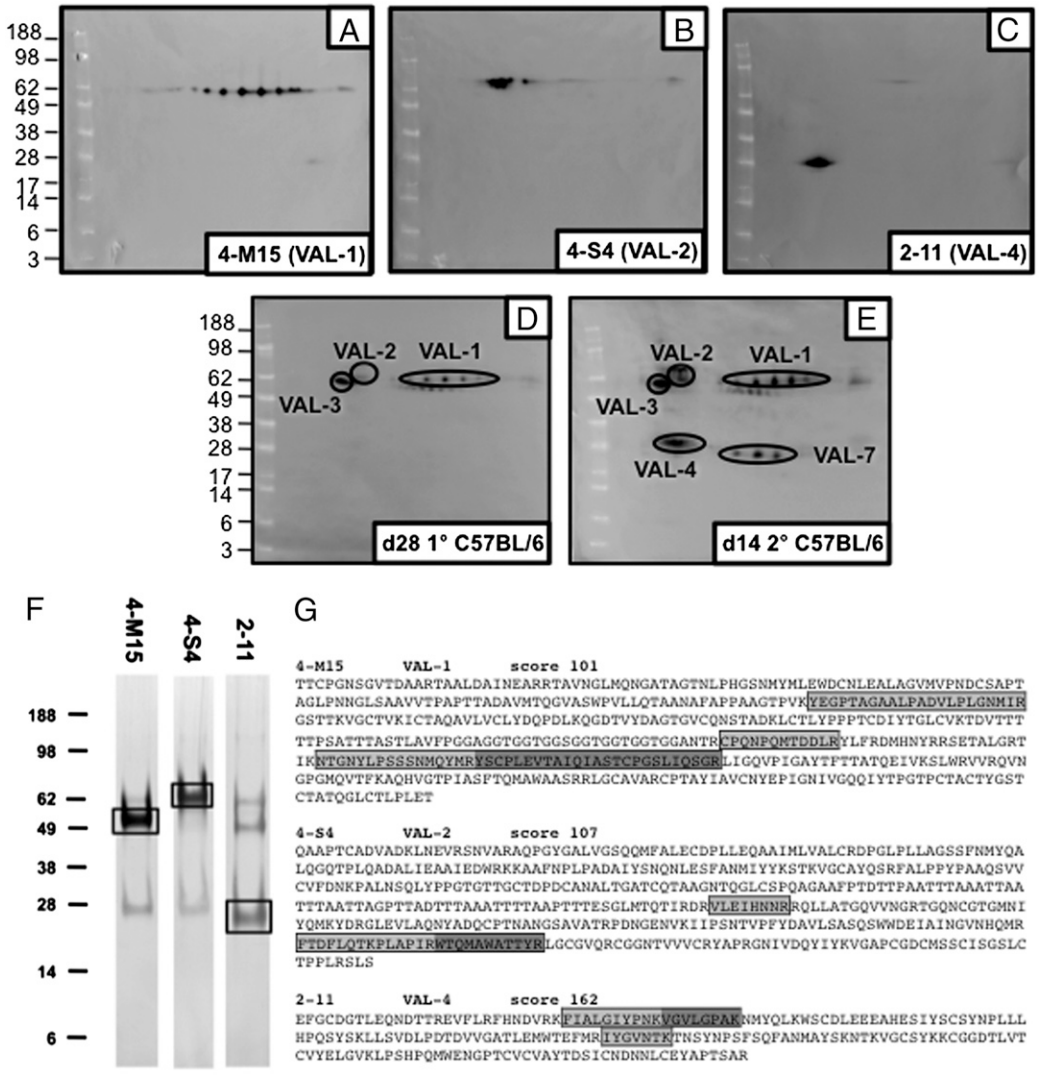

FIGURE 5. Conformation-dependent IgG1 Abs primarily target VAL Ags. *A–E*, Immunoprecipitation of biotin-labeled HES by the indicated mAb or polyclonal infection sera, separated by two-dimensional SDS-PAGE, and visualized by Western blotting with streptavidin–HRP. *A*, Anti–VAL-1 mAb 4-M15. *B*, Anti–VAL-2 mAb 4-S4. *C*, Anti–VAL-4 mAb 2-11. *D*, Primary day 28 C57BL/6 polyclonal infection serum. VAL-1, VAL-2, and VAL-3 are indicated. *E*, Secondary day 14 C57BL/6 polyclonal infection serum. Postions of VAL-4 and VAL-7 are indicated, as well as VAL-1, VAL-2, and VAL-3 as above. *F*, One-dimensional SDS-PAGE of immunoprecipitated (unlabeled) HES proteins using bead-conjugated mAb as indicated; boxes indicate segments eluted for mass spectrometric analysis. Additional bands likely reflect H and L chains of mAb leaching from beads. Molecular mass markers are indicated in kilodaltons. *G*, Peptides of VAL-1, VAL-2, and VAL-4 matched by mass spectrometry indicated by boxes. Mascot scores are also shown.

IgM Abs recognize a common O-linked glycan epitope on VAL glycoproteins

Despite being bound by several IgM monoclonals (Fig. 3*C*), the target Ags VAL-1, VAL-2, and VAL-5 show only limited amino acid homology (14.9% identity, 23.5% similarity; Supplemental Fig. 1), and reactivity is heat stable (Fig. 4*B*). We therefore evaluated the possibility of a carbohydrate nature of the target epitope(s). We also noted that the Ags' predicted molecular masses, based on primary amino acid sequences, were 10–39 kDa lower than their observed gel migration, yet each contained only a single potential N-linked glycosylation site (Fig. 6*A*). However, they encoded abundant serine and threonine residues in a central domain, which were predicted to be O-glycosylated by the NetOGlyc 3.1 program (Fig. 6*A*, Supplemental Fig. 2) (43). This contrasts with another secreted VAL protein of similar abundance, VAL-3 (Fig. 2*A*), which does not appear to be recognized by IgM Abs, lacks predicted O-glycosylation sites (Supplemental Fig. 2), and migrates on two-dimensional gels in a manner consistent with its predicted molecular mass (Figs. 3*A*, 6*A*).

To first confirm that glycan A is carried on VAL-1 and VAL-2, we showed that the anti-glycan A mAb 13.1 bound to VAL-1 and VAL-2 on Western blots following their affinity purification using IgG1 mAbs to the conformational epitopes of the VAL Ags (Fig. 6*B* and data not shown). Comparison with the profile of immunoprecipitated VAL-1 Ag (Fig. 5*A*) indicates that only three of the six VAL-1 spots react with the anti-glycan A mAb, as is also evident from Fig. 3*B*.

To then investigate the potential role of antigenic carbohydrates, we used both enzymatic and chemical deglycosylation strategies. When HES was pretreated with PNGase F to remove N-linked carbohydrates, small mobility shifts were evident in silver-stained gels (Fig. 6*C*) and Western blots (Fig. 6*D*), indicating the removal of *N*-glycans, yet polyclonal sera and IgM mAb reactivity remained intact (Fig. 6*D*, 6*E*). Similarly, the dominant Ag is unlikely to be an PNGase F-resistant *N*-glycan with a core α1,3-fucose, because PNGase A treatment of tryptic HES peptides failed to ablate either polyclonal or mAb binding (Supplemental Fig. 3). Furthermore, mass spectrometric analysis identified a number of peptides containing unconjugated N-glycosylation sites (NxS/T), calling into question whether these are used in the VAL proteins of this species (data not shown).

To assess the role of *O*-glycans, HES was treated with TFMS, which removes both *N*- and *O*-glycans. Such chemical deglycosylation resulted in mobility shifts of silver-stained HES bands, indicative of glycan removal, comparable to that seen with a control glycoprotein, RNase B (Fig. 7*A*). Importantly, TFMS treatment ablated both anti-VAL IgM mAb and polyclonal infection sera recognition of HES (Fig. 7*B*, 7*C*), whereas TFMS-treated HES retained reactivity with an anti-protein Ab, raised against recombinant *H. polygyrus* calreticulin, as well as polyclonal serum from rats immunized with HES (Fig. 7*D*). These data therefore indicate that the dominant and persistent IgM Ab response is to an O-linked glycan shared by several polypeptide secreted Ags of *H. polygyrus*. The status of the HM-65 Ag is less clear; although PNGase F treatment ablates binding of anti-glycan B 14.3 mAb to the 65-kDa component but not the high molecular mass complex, the reverse is true for TFMS (Figs. 6*D*, 7*B*). Because the 65-kDa Ag is less abundant, the overall reactivity of 14.3 to TFMS-treated HES is reduced ~10-fold but not abolished (Fig. 7*E*). Thus, a similar or identical epitope is conjugated through different linkages to different carrier macromolecules. It was also noted that TFMS does not remove the PC moiety from HM-65 (data not shown), indicating that a different linkage is used in *H. polygrus* from that described through N-linked glycans for *Acanthocheilonema viteae* (44).

A

	Predicted Mol.wt (–SP)	Observed Mw (kDa)	Discrepancy (kDa)	*N*-Glyco	*O*-Glyco
VAL-1	46.8	56.5	9.7	1	18
VAL-2	47.8	68.0	10.2	1	26
VAL-3	49.4	53.5	4.1	1	2
VAL-5	50.3	89.5	39.2	1	30

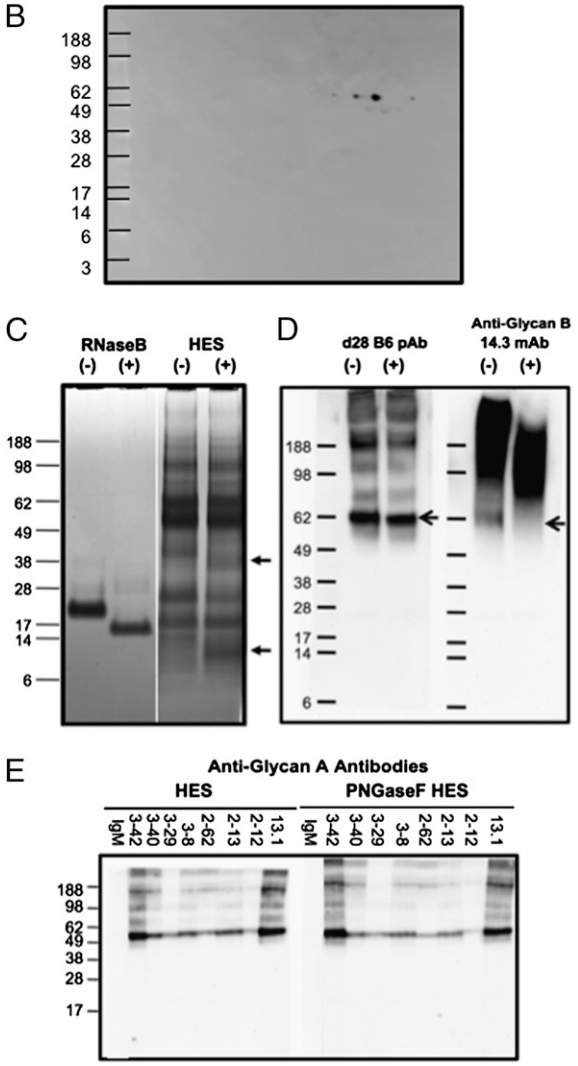

FIGURE 6. The immunodominant IgM target is not an N-linked glycan. *A*, Molecular mass discrepancies between the predicted molecular mass of indicated VAL proteins, based on amino acid sequence and observed migration on two-dimensional gels. Mature molecular mass is that predicted without the signal peptide (SP) sequence, predicted N-glycosylation sites are defined as N(X)S/T, and O-glycosylation sites were predicted using NetOGlyc version 3.1 (43). The proportion of predicted sites used is not known. *B*, Western blot of anti–VAL-1 mAb (4-M15)-precipitated Ag separated on two-dimensional SDS-PAGE and probed with anti-glycan A mAb (13.1). *C*, Silver-stained one-dimensional SDS-PAGE of PNGase F-treated (+) or mock-treated (−) RNase B control glycoprotein and HES. *D*, One-dimensional Western blot of HES with or without PNGase F treatment using day 28 C57BL/6 polyclonal sera and anti-glycan B (HM-65) mAb 14.3. Band shifts associated with enzymatic removal of *N*-glycans are arrowed. *E*, One-dimensional Western blots of HES with or without PNGase F treatment using anti-glycan A mAbs. IgM denotes an anti-DNP IgM mAb negative control. Molecular mass markers are indicated in kilodaltons

Glycans A and B as well as VAL-1 and VAL-4, but not VAL-2, are expressed by tissue-phase larvae

Upon infection of mice, *H. polygyrus* larvae first invade the submucosal tissue of the intestinal tract, molt twice (from L3 to L4

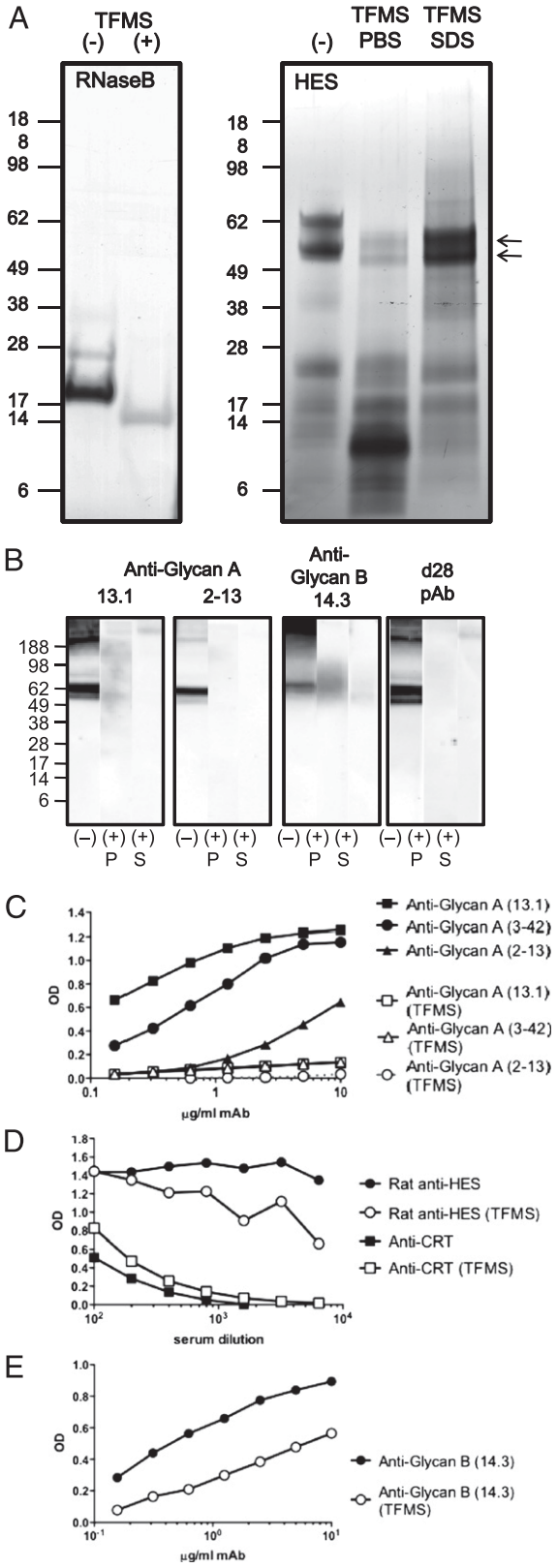

FIGURE 7. Chemical deglycosylation ablates anti-glycan A IgM Ab binding and reduces anti-glycan B binding to HM-65. *A*, Silver-stained one-dimensional SDS-PAGE of TFMS-treated (+) or mock-treated (−) HES and RNase B control glycoprotein. TFMS-treated HES was separated into PBS and SDS-soluble fractions. *B*, One-dimensional Western blots of mock-treated and TFMS-treated HES probed with anti-HES mAbs against glycans A and B and day 28 C57BL/6 polyclonal antiserum. *C*, ELISA of TFMS-treated (open symbols) and mock-treated (solid symbols) HES, used to measure binding of anti-glycan A mAb 13.1 (squares), 2-13 (triangles), and 3-42 (circles). *D*, ELISA of TFMS-treated (open symbols) and mock-treated (solid symbols) HES probed with polyclonal rat anti-HES serum (circles) or rat Ab to recombinant *H. polygyrus* calreticulin (Hp-CRT; squares). *E*, ELISA of TFMS-treated (open symbols) and mock-treated (solid symbols) HES probed with the anti-glycan B (HM-65) mAb 14.3. The PBS-soluble fraction of TFMS was used for ELISA in *C–E*. Molecular mass markers are indicated in kilodaltons.

and then adult), emerging 8 d later into the lumen of the small intestine (1). As immunity to challenge infection is directed, in part at least, at the tissue-phase larvae (3), we investigated whether the Ags defined from adult parasites are also expressed by fourth-stage larvae (L4) recovered from the submucosa at day 5 post-infection. Larvae were cultivated to yield ES Ags, which were probed with each of the specific Ab reagents. Polyclonal anti-HES antiserum reacted strongly with L4 ES (Fig. 8*A*); positive reactions to L4 ES were also seen with mAbs to glycan A (Fig. 8*B*) and glycan B (Fig. 8*C*), although in both cases levels were lower than with HES. Interestingly, among the VAL Ags, it was found that although VAL-1 (Fig. 8*D*) and VAL-4 (Fig. 8*F*) were present at similar levels in HES and L4 ES, VAL-2 was absent from the larval stage (Fig. 8*E*).

Glycan A is strongly represented on the adult cuticle, whereas glycan B is a somatic Ag

To localize glycan A and B epitopes within the adult parasite, we probed intact worms and sections by immunofluorescent microscopy. In sections, anti-glycan A mAb bound the worm surface, highlighting the longitudinal ridges of the cuticle (Fig. 9*A*). Anti-glycan A Ab also bound to the surface of intact adults in a pattern that similarly emphasized the ridges (Fig. 9*B*). At higher magnification, anti-glycan A Ab was seen to stain an ordered array of epitopes organized longitudinally along the cuticular furrows (Fig. 9*C*). In contrast, anti-glycan B Abs failed to bind to intact worms (Fig. 9*D*) while reacting strongly to somatic constituents in cross-sections (Fig. 9*E*); in particular, no cuticular binding was observed with anti-glycan B Ab.

To determine the macromolecules to which cuticular glycan A is conjugated, we surface radiolabeled adult worms and used mAbs to immunoprecipitate glycan A-bearing Ags. As shown in Fig. 9*F*, this procedure indicates that glycan A is expressed on at least four different molecular mass species, with a 180-kDa band predominating (Fig. 9*F*); only a small proportion of ~55-kDa VAL proteins are similarly immunoprecipitated. The conclusion that most surface glycan A is not borne on VAL proteins is supported by studies reported elsewhere that anti–VAL-1 and –VAL-2 mAbs bind only to localized areas of the cuticle (34). In contrast to glycan A, the glycan B-specific Abs were unable to immunoprecipitate radiolabeled surface components (Fig. 9*F*). A remarkable degree of cross-reactivity between ES and surface proteins was indicated as a polyclonal sera raised against HES was able to precipitate essentially all radiolabeled surface components (Fig. 9*F*).

Vaccination with HES confers protection against challenge whereas passive immunization with anti-HES mAb does not

Given the protective ability of VAL family members such as ASP in related helminth infections (45), we determined whether passive immunization with the anti-VAL protein and glycan mAbs were able to confer protection against challenge infection. We first wished to verify that adult worm-derived HES, in which VAL proteins are among the major Ags, could effectively vaccinate against challenge with larval parasites, as negative results have been reported in the literature (46). We found, however, that HES

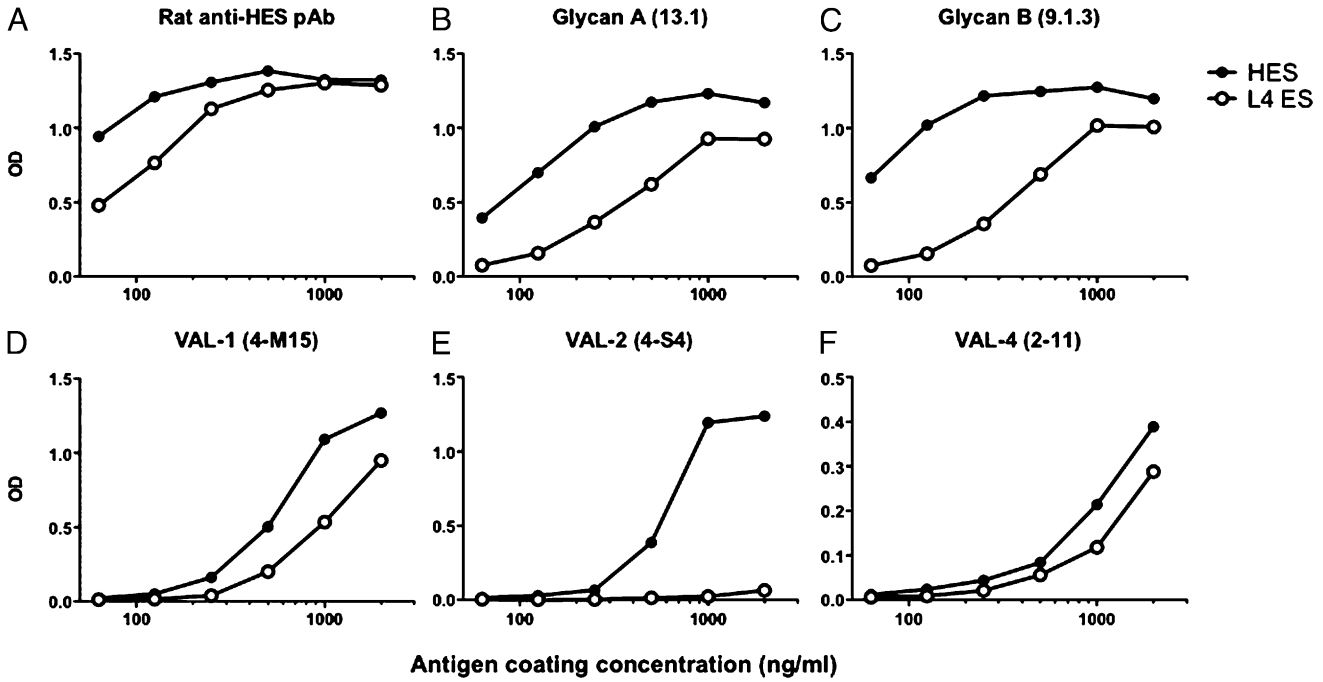

FIGURE 8. Monoclonal binding to ES from the tissue-phase L4 larvae show expression of glycans A and B, VAL-1 and VAL-4, but not VAL-2. *A*, ELISA reactivity of polyclonal rat anti-HES binding to plates coated with range of concentrations of adult HES (solid symbols) or L4 ES (open symbols) *B*, As above, with anti-glycan A mAb 13.1. *C*, As above, with anti-glycan B mAb 9.1.3. *D*, As above, with anti–VAL-1 mAb 4-M15. *E*, As above, with anti–VAL-2 mAb 4-S4. *F*, As above, with anti–VAL-4 mAb 2-11.

vaccination with alum adjuvant induced a potent humoral response, with titers of anti-HES IgG1 comparable to those seen following secondary infection (data not shown). Moreover, vaccinated animals had greatly reduced egg counts at day 14 than animals immunized with PBS-adjuvant alone (Fig. 10*A*), whereas by day 28, HES-immunized animals showed no fecal eggs and had expelled all adult worms (Fig. 10*A*, 10*B*). Thus, immune responses against adult secretions conferred highly effective and significant protection against larval challenge.

We next performed passive immunization experiments with each of the defined specificity mAbs given throughout the infection period. Using either 0.2 or 1 mg doses of mAb every 2–3 d, mice given anti–VAL-1, –VAL-2, or –VAL-4 IgG1 Abs showed no diminution in egg counts at day 14 (Fig. 10*C*) or day 28 (Fig. 10*D*) of infection, and indeed, anti–VAL-4 recipients showed elevated egg numbers in two independent experiments ($p < 0.05$ compared with recipients of MOPC31C). Furthermore, none of the anti-VAL mAbs induced worm expulsion as measured at day 28 (Fig. 10*E*).

In the same experiments, we also tested IgG1 anti-glycan B mAb for ability to passively protect recipient mice; however, as shown in Fig. 10*C–E*, this Ab also failed to reduce egg numbers or elicit expulsion of adult worms. Finally, IgM Ab against glycan A was tested, because no class-switched Abs of this specificity were observed. As with the other mAbs, anti-glycan A did not protect against egg production (data not shown) or worm persistence (Fig. 10*F*).

Discussion

The model system of *H. polygyrus* captures many essential characteristics of the gastrointestinal nematode infections that are highly prevalent in human and animal populations (47, 48). The parasite establishes a chronic infection, driving regulatory T and B cell subsets within a Th2-dominated environment (5, 25, 49–51) and altering innate populations such as dendritic cells (52, 53) and macrophages (54, 55). Immunity to *H. polygyrus* is slow to develop, particularly in the genetically most susceptible hosts (14), but both B cells and the Abs they produce are important constituents of the protective immune response (4, 15, 16). In this study, we aimed primarily to define the Ags of adult parasites recognized by host serum Abs and secondarily to address whether those Ab specificities serve a protective function in the host–parasite relationship.

Previous antigenic analyses of *H. polygyrus* have involved a mixture of approaches and relatively simple characterization such as one-dimensional SDS-PAGE or column fractionation (32, 33, 56) or have investigated individual gene products that are postulated to play a role in immune recognition (26, 31, 57, 58). We have adopted in this study a more global approach to identify the major Ags recognized during primary infection, which we show are well represented in HES, although not in somatic extracts, presumably reflecting the fact that the immune system is exposed in a more continuous fashion to the secreted products of a luminal-dwelling live parasite. Indeed, we have detected glycan A in the serum of 7-d infected mice, demonstrating that products of a gastrointestinal parasite can disseminate to distant sites (J.P. Hewitson, unpublished observations). Ongoing work has also revealed that T cell Ags, driving the secretion of Th2 cytokines, are enriched in HES compared with somatic extract in a similar manner to serological Ags (J.P. Hewitson and K.J. Filbey, unpublished observations).

Murine Ab responses are known to be predominantly IgG1 with primary reactivity to secreted Ags in the 50- to 70-kDa range (14, 59). These Ags correspond to those we have now defined as VAL-1, VAL-2, and VAL-5 and appear likely to represent the products isolated from HES by Monroy et al. (57) to achieve a 40% reduction in egg production following vaccination. Interestingly, these glycoproteins bear a conserved antigenic carbohydrate we have termed glycan A, the structural analysis of which is now under way. Glycan A is also strongly associated with the cuticular surface of the adult worm and is partly responsible for the extensive degree of antigenic sharing between the secretions and

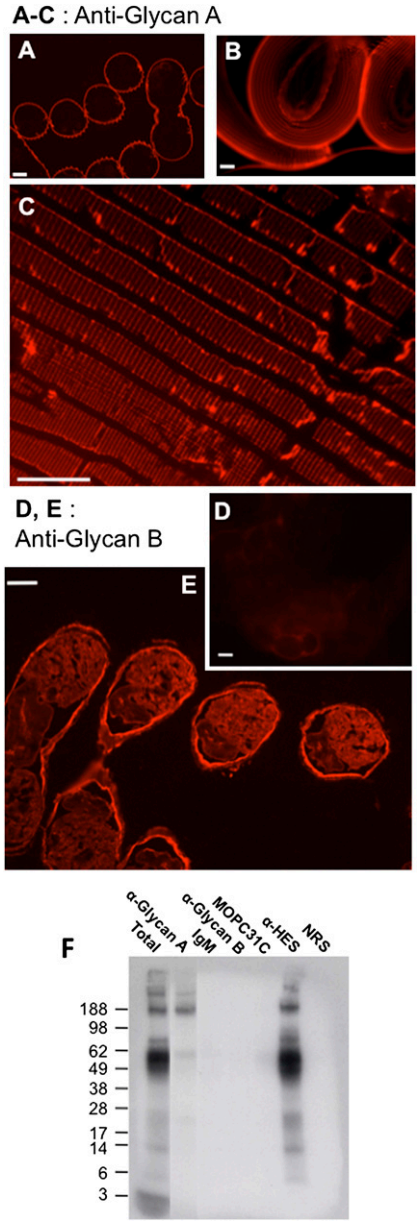

FIGURE 9. The immunodominant glycan A is present on the surface of adult worms. *A*, Binding of anti-glycan A mAb 13.1 to transverse sections of adult *H. polygyrus.* The corrugated cuticular ridges are visible. *B*, Binding of anti-glycan A mAb 13.1 to the surface of intact adult *H. polygyrus. C*, Binding of anti-glycan A mAb 13.1 to ordered structures within cuticular furrows on the surface of *H. polygyrus*. *D*, Failure of anti-glycan B mAb 14.3 to bind to intact worms. *E*, Binding of anti-glycan B mAb 14.3 to muscle layer and other structures in transverse sections of adult worms. *F*, SDS-PAGE of ^{125}I surface-labeled adult proteins immunoprecipitated with anti-glycan A mAb 13.1, revealed by autoradiography; similar analysis with anti-glycan B mAb 9.1.3 is also shown. Control IgM and IgG1 (MOPC31C) proteins are shown, as well as polyclonal rat anti-HES and normal rat serum (NRS). Scale bars (*A–E*), 100 μm. Molecular mass markers are indicated in kilodaltons.

surface of adult *H. polygyrus*, as reported by earlier investigators (33). This may indicate that HES, and the VAL components in particular, are shed from the surface of the worm or alternatively that molecules secreted in HES remain associated in some form with the parasite cuticle.

The VAL glycoproteins represent the immunodominant target of both IgM (against glycan A) and the class-switched IgG1 response (against a conformational epitope, presumably the protein backbone). Most VAL proteins from other species have been reported to be glycosylated based on discrepancies between predicted and observed molecular mass [e.g., *Ancylostoma caninum* VALs (60)], although only *N*-glycans have so far been identified [on a VAL protein from the cattle nematode *Ostertagia ostertagi* (61)]. In this article, we show that a subset of *H. polygyrus* secreted VAL proteins are decorated with highly antigenic *O*-glycans, which are most likely concentrated in a serine/threonine-rich tract linking two conserved sperm coating protein (cd05380) domains. Similar stretches of predicted O-glycosylation can be observed in other nematode VAL proteins, including *Haemonchus contortus* Hc40 (accession number AAC03562; http://www.ncbi.nlm.nih.gov/), *Cooperia punctata* ASPs (AAK35199 and AAK35187), and *A. caninum* ASP-4 and ASP-6 (AAO63576 and AAO63578). It is also important to note that the VAL proteins themselves, rather than only their associated glycans, are likely to play a key role in host–parasite interactions, as *H. polygyrus* secretes a number of abundant nonglycosylated VAL proteins such as VAL-3 (34).

The nematode cuticle is an extracellular matrix assembled from specialized collagens and cuticlins (62), with sugar components that may vary greatly between species. For example, *Trichinella spiralis* conjugates a unique immunodominant glycan with a terminal tyvelose sugar onto multiple peptide backbones (63), providing a target for protective Abs (64, 65), whereas larvae of *Toxocara canis* (which invade the intestinal tract and migrate in tissues) release two unusually methylated and highly antigenic O-linked trisaccharides (66–68). Larvae of *Trichostrongylus colubriformis*, which, like *H. polygyrus*, is a member of the Trichostrongylid family, express a protease-resistant carbohydrate Ag that may act as the target of protective immunity (69). Another antigenic moiety closely related with helminth parasites is phosphorylcholine, which may be associated with high molecular mass proteoglycan-like molecules (30) or individual proteins that resolve on SDS-PAGE (41). Interestingly, both appear to be the case for HES in which PC is present on a high molecular mass product that does not stain for protein, as well as a 65-kDa component. Most intriguingly, mAbs to this combination (termed HM-65) do not react to PC itself, indicating that a distinct but as yet uncharacterized structure (provisionally named glycan B) is expressed. It remains possible that "glycan B" is not a true carbohydrate but a small haptenic group similar to diethylaminoethanol that is found in ES Ags of the filarial nematode *Litomosoides sigmodontis* (70). Hence, although we do not yet know the structural nature of glycans A and B, it is clear that *H. polygyrus* is not unusual in presenting extensive and immunodominant nonprotein specificities to the mammalian host.

Abs may exert a protective effect by several pathways (17); in particular, they may impede growth and migration during the histotrophic larval phase (20, 71), possibly by neutralizing key ES products (6), and may target the exposed epitopes on the surface of adult worms during the luminal phase. A major objective in studying Ab–Ag interactions in *H. polygyrus* is therefore to address whether particular specificities can confer immunological protection against infection. It is known that Abs play an important role in protective immunity to this parasite, as B cell-deficient animals suffer impaired immunity against secondary infection (4, 15, 16) as a result of absence of Ab as well as B cell participation in the cellular response. In tests of polyclonal serum Abs, the passive transfer of secondary infection sera can protect against challenge infection (15, 72), and this was associated with the IgG1 fraction (19). Although attempts to transfer immunity with primary infection sera have been less successful (15), purified IgG1 can reduce worm burdens and lead to stunting of adult parasites

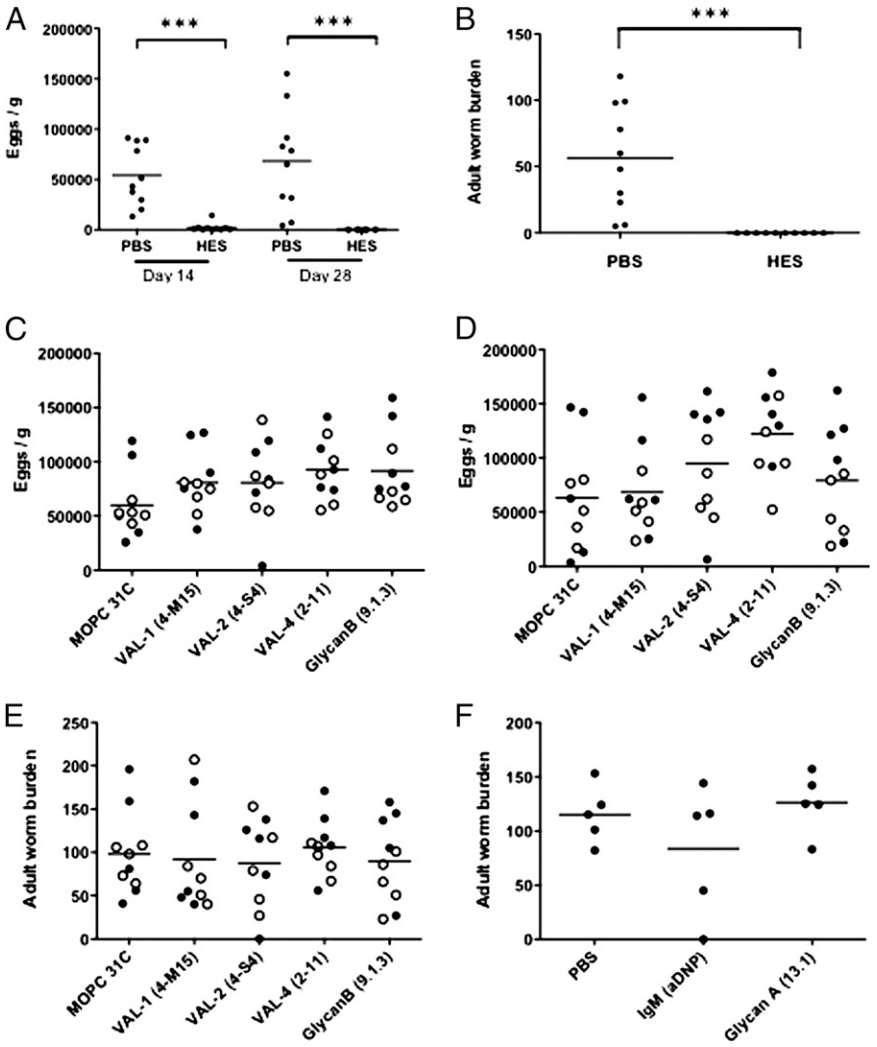

FIGURE 10. Vaccination with adult HES confers sterile immunity to challenge, but passive immunization with mAbs from infected mice does not lead to worm expulsion. *A*, Fecal egg counts at 14 and 28 d postinfection in HES-alum vaccinated mice compared with PBS-alum alone. Each symbol represents a single mouse, and data shown are combined from two independent experiments. ***$p < 0.0001$. *B*, Adult worm burdens at 28 d postinfection in HES-alum vaccinated mice compared with PBS-alum alone. Each symbol represents a single mouse, horizontal bars denote mean values, and data shown are combined from two independent experiments. ***$p < 0.0001$. *C* and *D*, Fecal egg counts at 14 d (*C*) and 28 d (*D*) postinfection in mice receiving passive immunizations of anti-HES IgG1 mAbs or of MOPC31C control IgG1 Ab. Each symbol represents a single mouse, horizontal bars denote mean values, and data shown are combined from two independent experiments using 0.2 mg (open symbols) or 1 mg (closed symbol) mAb for each dose. *E*, Adult worm burdens at day 28 postinfection in the experiments shown in *C* and *D*. *F*, Adult worm burdens at day 28 postinfection in mice passively immunized with PBS alone, control anti-DNP IgM, or IgM anti-glycan A mAb 13.1. i.v. Each symbol represents a single mouse, and horizontal bars denote mean values; similar results were obtained in an independent experiment following i.p. delivery of mAb. Mice receiving i.p. injections of IgG1 Abs were assayed 24 h following the first transfer and were found to have specific anti-HES serum Abs equivalent to between 217 and 633 μg/ml mAb (data not shown).

(20). This may imply that the efficacy of secondary serum Abs over primary sera reflects elevated titers of anti-worm Abs, rather than any difference in specificity induced by repeat exposure.

We therefore tested each of the mAb types generated in this study for their ability to confer protection by passive transfer. However, none exerted any effect on immunity. As we transferred considerable quantities of each, and these mAbs represent the major specificities present in primary infection sera, our data instead argue that the failure of primary sera to protect does not reflect a quantitative insufficiency in terms of concentration but either an inadequate affinity as a result of limited affinity maturation or, most interestingly, the absence of key new specificities that may arise only after multiple infections. The failure of mAb-mediated passive immunization contrasts with the sterile immunity generated following vaccination with HES that generates circulating anti-HES titers comparable to those seen following secondary infection. We also therefore examined the Ab profile following secondary infection, which is broadly similar to the primary but contains some additional specificities (such as the single-domain VAL-7 Ag). Future work will examine whether these are more effective targets of protective immunity. A further possibility to be tested is that combinations of Abs, for example, against each of the related VAL proteins, are required to neutralize a common function and that the full range of anti-VAL Abs are only generated through secondary infection.

An intriguing finding of this study is that the dominant anti-glycan A response, which is rapidly and extensively stimulated by *H. polygyrus*, shows little protective capacity. This epitope may thus represent an example of a decoy Ag that is elaborated by the parasite to distract immune responses without risk of it inducing a lethal attack on the worm. It is also notable that despite strongly binding the adult worm surface, anti-glycan A Abs are not protective in vivo; this could reflect their restriction to IgM, suggesting that in the absence of class-switching Abs may not gain access to the intestinal sites of infection. The lack of other isotypes even after 28 d of infection suggests a deficiency in T cell help, potentially at the level of the TFH. As it is known that there are abundant IL-4–producing TFH in the draining MLN of *H. polygyrus* mice early in primary infection (73), it is surprising that no IgG response to glycan A is mounted, suggesting that this specificity is recognized and/or processed in an unusual manner.

The longevity of parasites can be attributed to their ability to evade or divert host immunity (74), and hence, the molecular basis of immune attack is of paramount interest. Our understanding of immune interactions with gastrointestinal helminths is primarily at the level of effector cell populations (39, 75) rather than identification of target molecules. At this time, the definition of parasite molecules in this important model of chronic gastrointestinal infection will provide an essential platform both to analyze the recognition of Ags and to identify the parasite products (proteins and sugars) that can modulate host immunity and facilitate protection (22). In this article, we have accordingly moved our definition forward from using crude parasite extracts to the more

antigenic and less complex HES, and finally to individual antigenic species, so that future work can study defined glycans and recombinant proteins. We have also established that most of the prominent Ags are secreted not only by the luminal-dwelling mature adult worms but also by the histotrophic larval stage, which is considered to be a major target of protective immunity. We can now begin to investigate how and where Abs act (21), the relative importance of functional neutralization and the recruitment of host effector cells, and the lethal mechanisms that achieve sterilizing immunity against intestinal helminths.

Disclosures

The authors have no financial conflicts of interest.

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